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(54) Title: ASSAY FOR MULTIDRUG RESISTANCE

(57) Abstract

A reliable and quantifiable measure of multidrug resistance based on a functional assay of fluorescent dye exclusion from cells. As herein disclosed, the selection of a fluorescent substrate having slow transmembrane movement results in an improved assay overcoming many of the shortcomings of existing MDR assays.

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ASSAY FOR MULTIDRUG RESISTANCE

FIELD OF THE INVENTION

The invention relates to the use of fluorescent markers distinct in their slow movement through phospholipid bilayers for the detection and quantification of multidrug resistance and its modulation. More particularly, it provides a functional assay for the level of multidrug resistance using fluorescent dyes with slow transmembrane movement.

BACKGROUND OF THE INVENTION

The use of anticancer drugs in the therapy of various human malignancies is often hampered by inherent as well as acquired multidrug resistance (MDR), a phenomenon characterized by the resistance of the tumors to several structurally and functionally distinct hydrophobic chemotherapeutic compounds. Multidrug resistance is related to the reduced cellular accumulation of these drugs and to the overexpression of a transporter protein known as P-glycoprotein (Pgp), or gp170, which possesses an ATPase activity, and functions as an energy-dependent extrusion pump which expels hydrophobic cytotoxic agents from MDR cells (Gottesman and Pastan 1993, *Annu. Rev. Biochem.* **62**, 385-427).

Expression of mammalian Pgp is not limited to MDR tumor cells but is also found in a number of normal tissues. Little is known about the function of physiologically expressed Pgp and even less about its putative natural substrates. The localization of Pgp to the bile canaliculus, small intestine, colon and proximal renal tubules led to the suggestion that Pgp is involved in the excretion of xenobiotics (Sugawara et al. 1988, *Cancer Res.* **48**, 1926-1929). A possible physiological role of Pgp is protection of the brain by extrusion of neurotoxins across the blood-brain barrier. Pgp is highly expressed in brain capillary endothelial cells (Cordon-Carlo, et al., 1990, *J. Histochem. Cytochem.* **38**, 1277-1287), and prevents MDR-type drugs from accumulating in the brain. Schinkel et al. (*Cell*, 77, 491-502, 1994) have generated mice with a homozygous disruption of the *mdr*1a gene, which encodes Pgp. The absence of Pgp from the blood-brain barrier in these mice resulted in elevated MDR-type drugs in their brain and a hypersensitivity to the centrally neurotoxic pesticide, ivermectin.

The rate of free diffusion of solutes across membranes has been correlated with their hydrophobicity and size. In this respect, the various MDR-type anticancer drugs are hydrophobic and thus expected to be membrane permeable. Yet, Pgp with its limited drug transport rate (Kcat=900 min^{-1}) is not expected to keep pace with the rapid diffusional influx of these MDR-type drugs (Borgnia et

al. 1996, *J. Biol. Chem.* **271**, 3163-3171), whereas in point of fact Pgp succeeds in extruding these drugs from the cells. This paradox suggests the possibility that despite their hydrophobicity, the various MDR-type drugs are actually not freely membrane permeable. Moreover, the requirement for an active efflux pump to mediate extrusion of xenobiotics from the body appears to conflict with the presumed membrane permeability of Pgp substrates.

This apparent paradox prompted the direct measurement of the transmembrane movement rate of various MDR substrates and modulators (Eytan et al. 1966, J. Biol. Chem. 271, 12897-12902). Fluorescence quenching studies showed that the transmembrane movement of rhodamine 123 proceeded with a life-time of 3 min. As studied by binding studies, the transmembrane movement of established MDR-type anticancer drugs, including doxorubicin, vinblastine and taxol, occurred at a comparable rate to that of rhodamine 123. These results suggest that anticancer drugs do not diffuse freely across membranes, thus allowing Pgp to efficiently exclude them from MDR cells. Thus, the efficiency of the Pgp-mediated exclusion of various drugs from MDR cells is governed by both its capacity to expel drugs and the transmembrane influx rate of the drugs.

Considerable effort has been expended on the development of methods to detect P-glycoprotein-associated multi-drug resistance in patient's tumors (reviewed by Beck et al. 1996, *Cancer Res.* **56**, 3010-3020). These assays are generally unsatisfactory in that they suffer from poor sensitivity and therefore yield highly variable results, particularly when dealing with lower levels of MDR. Furthermore, there is generally inconsistent correlation between the clinical outcome of chemotherapy and the assessment of MDR by currently available assays.

Various specific assays for multidrug resistance or for Pgp are known in the art, including assays based on monoclonal antibodies to Pgp, as disclosed in US Patents 5,369,009 and 5,434,075, or an assay based on the correlation of MDR with an alkaline shift in the intracellular pH of cells, as disclosed in WO 95/21381.

It is also generally known that the efflux activity of Pgp can be measured utilizing a variety of fluorescent substrates, including the naturally fluorescent drug substrates such as daunomycin or doxorubicin. Another class of substrates includes a variety of fluorescent dyes including rhodamine dyes (Neyfakh, *Exp. Cell Res.* **174**, 168-176, 1988; Homolya et al. *J. Biol. Chem.* **268**, 21493-21496, 1993; Wall et al. *Eur. J. Cancer* **29A**, 1024-1027, 1993). The sensitivity of these assays is generally poor, and can be utilized only for the qualitative assessment of whether a given population of cells is MDR or not. Efforts have been made to find

novel dyes with improved sensitivity in flow cytometric dye efflux assays for the identification of MDR (Frey et al., *Cytometry* **20**, 218-227, 1995).

Nowhere in the background art is it taught or suggested that the selection of fluorescent substrates having slow transmembrane movement for dye efflux assays results in greatly improved sensitivity of the assays.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide a functional assay suitable for detection and quantification of multidrug resistance or its modulation. According to the present invention a simple, reliable and quantifiable assay is provided, based on the correlation between the passive transmembrane movement rate of MDR substrates and the ability of Pgp to extrude them from MDR cells.

As herein disclosed, the selection of a fluorescent substrate having slow transmembrane movement rate results in an improved assay overcoming many of the shortcomings of existing MDR assays. Furthermore, these substrates can be used for the screening and selection of efficient MDR modulators having high transmembrane movement rates, thereby predicting the ability of these molecules to overcome MDR.

Methods are provided for the screening of normal and malignant cells for pre-existing MDR, for screening for the onset of MDR, and for screening of modulators designed to overcome MDR.

According to the present invention an assay for multidrug resistance is provided, comprising exposing cells to a fluorescent dye having slow transmembrane movement rate, and quantitatively determining the proportion of cells stained by the dye and the relative intensity of staining of individual cells.

According to another embodiment of the invention a dye having slow transmembrane movement rate is used to assay the usefulness of compounds which are candidates to act as MDR modulators, capable of reversing MDR.

According to the principles of the present invention, a fluorescent dye having relatively slow transmembrane movement rate, with a half equilibration period exceeding 10 minutes at room temperature, is preferred. More preferred are dyes having transmembrane movement rate with a half equilibration period exceeding 15 minutes at room temperature. Estimation of transmembrane movement is expressed herein as the equilibration rate throughout multilamellar vesicles as defined herein.

A most preferred embodiment according to the present invention utilizes rhodamine or rosamine analogs having slow transmembrane movement including tetramethylrosamine also denoted herein as TMR.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 presents flow cytometric histograms comparing cellular uptake of tetramethylrosamine and rhodamine 123. Wild-type cells of the AA8 cell line, as well as itssublines, T19, and Emt^{R1} cells were incubated for 1 h in a medium containing either 0.2 or 10 μM of either tetramethylrosamine or rhodamine 123. Mean linear red fluorescence per cells is displayed in a 1024 channel abscissa. The autofluorescence of Emt^{R1} cells obtained in the absence of the dye served as a control. The effect of MDR-modulators (10 μM) verapamil and cyclosporin A were examined in T19 cells (the second panel).

FIG. 2 demonstrates accumulation of rhodamine dyes in CHO MDR-cells. The accumulation of the various rhodamine dyes (at 10 μM concentration) in AA8, T19, and Emt^{R1} cells was determined as described in Fig. 1. The median fluorescence of T19 (empty bars) and Emt^{R1} (filled bars) cells are plotted (SD, n=4) relative to the median fluorescence of the wild type AA8 cells.

FIG. 3 demonstrates dose dependence of TMR accumulation in an ovarian carcinoma cells and their MDR subline. Parental 2780 (squares) and MDR 2780^{ADR} cells (circles) were incubated for 1h 37 °C in the presence of TMR at various concentrations and the fluorescence of cells was determined by flow cytometry. Fluorescence values were corrected for the autofluorescene of unstained cells.

FIG. 4 demonstrates binding of rhodamine dyes to MLV. The extent of dye binding to MLV was determined as described under "Experimental Procedures". The extent of dye binding is expressed as binding coefficients equivalent to the dye concentrations in the membrane matrix divided by the concentration in the medium.

FIG. 5 demonstrates equilibration kinetics of the various rhodamine dyes with MLV. The binding kinetics of the rhodamine dyes was determined as described under "Experimental Procedures". The time periods required to achieve half maximal dye binding are presented in the figure.

FIG. 6 demonstrates effect of TMR and rhodamine 123 on ATPase activity of reconstituted Pgp. Pgp was extracted from Emt^{R1} cells and reconstituted into proteoliposomes. The ATPase activity of these proteoliposomes was determined following one hour incubation in the presence of various concentrations of either tetramethylrosamine (squares) or rhodamine 123 (circles). The basic Pgp ATPase activity, in absence of added dye, was equivalent to 1.15 μmoles Pi released min^{-1} mg^{-1} protein. The ATPase activity obtained in the presence of the dyes was divided by the value of the basic ATPase activity obtained in the absence of any exogenously added drug.

FIGs. 7a-b demonstrate effect of rhodamine dyes on ATPase activity of reconstituted Pgp. ATPase activity of reconstituted Pgp was determined as described in Fig. 4 in the presence of the various concentrations of the rhodamine dyes. The increase in ATPase activity (7a) measured in the presence of 50 μM dye was calculated relative to the basal activity. The concentration required to achieve half-maximal increase in activity (the apparent K_m) is presented in 7b.

FIG. 8 demonstrates hydrophobicity of the various rhodamine dyes. Dye samples (0.25 pmoles) were chromatographed on silica gel-coated plates with chloroform: methanol: water (190:30:1, v:v:v) as a developer. The R_f is correlated with the hydrophobicity of the dyes.

FIGs. 9a-b demonstrate TMR uptake into cells of a healthy donor and a CML patient. Frozen mononuclear (MN) cells from a healthy donor and the CML patient described in the "Examples" section were thawed and subsequently incubated for 1 hour at 37 °C with the nM concentrations of either TMR (9a) or rhodamine 123 (9b). The cells were washed and analyzed by flow cytometry.

FIG. 10 demonstrates TMR uptake into cells from a CML patient compared to healthy donors. Six separate samples were obtained from the same CML patient whose cells were analyzed in Figs. 9a-b and compared to samples obtained from fifteen healthy donors. All samples were frozen as described under "Experimental Procedures". Each CML sample was processed for TMR staining on a different day together with several samples from healthy donors. Uptake of rhodamine 123 amounts into five cell samples from the same CML patient (circles) and samples from six healthy donors (squares) was measured. The amount of TMR staining of the CML cells were significantly lower compared to the cells obtained from the healthy donors ($P<0.0005$ at TMR concentrations 2.5-2,500 nM). The best corresponding differences were obtained with 25-250 nM rhodamine 123, but were not statistically significant ($P=0.08$).

FIG. 11 demonstrates flow cytometric analysis of cells with functional TMR staining combined with anti-CD34 immunostaining. Frozen cell samples of the CML patient whose cells were analyzed in Figs. 9a-b were thawed as described under "Experimental Procedures". The cells were incubated for 1 hour in the absence or presence of 25 nm TMR and in the absence (control) or presence of either control IgG or anti-CD34.

FIG. 12 presents double dimension analysis of cells with TMR staining and anti-CD34. The experiment described in Fig. 11 was analyzed by gating dot-plots of cells treated with TMR and either control-IgG or anti-CD34 into dye-resistant cells and dye-sensitive cells, as shown in the figure for cells treated with TMR and anti-CD34. The gated cells were analyzed for the green fluorescence associated

with either control IgG or anti-CD34. Comparison of the fluorescence intensity of cells stained with anti-CD34 to the fluorescence associated with the same cells treated with control IgG allows easy identification of CD34-positive subpopulations.

FIG. 13 presents double dimension analysis of cells with TMR and anti-Pgp. An experiment similar to that described in Fig. 11 was repeated with cells from the same patient except that anti-Pgp and a corresponding control IgG were used instead of anti-CD34. The cells were analyzed as described in Fig. 12.

FIG. 14 demonstrates TMR staining of cells from newly-diagnosed CML patients. Frozen cells acquire from three CML patients, at the initial presentation (squares) and after a short course of chemotherapy (triangles), were thawed and stained with various TMR concentrations. The TMR uptake into these cells were compared to the average uptake of TMR into 15 healthy donors (circles). The TMR content of cells obtained from the CML patients and stained with 2.5 nM TMR was significantly lower than the dye uptake into similarly-treated cells obtained from healthy donors (P=0.0002). Likewise, the TMR content obtained from the same patients after treatment was significantly lower compare to cells collected from the patients at presentation (P=0.00008).

FIG. 15 demonstrates effect of freezing of cells on TMR staining. Cells from a healthy donor (triangles) and an ALL patient (squares and circles) were stained with TMR either as fresh samples (inverted triangles and circles) or after freezing and thawing (triangles and squares).

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides fluorescent dyes suitable for use in a reliable quantifiable assay of multi-drug resistance, selected on the basis of novel functional criteria. The principles of the assay are based on the functionality of dye extrusion or exclusion from the cells examined, rather than on the amount of Pgp protein, or other markers of the transformed MDR phenotype. The improvement lies both in this functional aspect of the assay, as well as in the high sensitivity of the assay due to the lowered background. These and yet other objects are accomplished by the selection of a fluorescent dye having the property of slow transmembrane movement. This property allows the MDR cells to extrude the dye efficiently, thereby providing a sensitive measure of the MDR activity in any given cell or cell population.

As used herein and in the claims the term "transmembrane movement" refers to the rate at which a compound is capable of crossing a membrane comprising a phospholipid bilayer, such as a cell membrane, as expressed in the

average residence time of a compound in a membrane monolayer prior to transfer across the membrane to the other monolayer.

As used herein and in the claims the term "multi-drug resistance" refers to the ability of a cell to extrude lipophilic cytotoxic drugs, exhibiting cross resistance with drugs included in the multi-drug resistance phenomenon, e.g., anthracyclines, Vinca alkaloids, epipodophyllotoxins, actinomycin D, taxoids, and hydrophobic peptides, such as gramicidin D, valinomycin, and dolastatin 10.

As used herein and in the claims the term fluorescent dye refers to any chemical compound having a chromophore capable of emitting light at a different visible wavelength from that at which it is excited.

As will be exemplified herein the present invention discloses the usefulness of particular fluorescent dyes in the assay of multi-drug resistance. The cells to be tested are exposed to a suitable fluorescent dye, selected according to the property of slow transmembrane movement, and the cells are then analyzed for the extrusion or exclusion of this dye from the cells. The dye in the cells can be analyzed according to principles known in the art. Most conveniently the cells can be so analyzed by cytometric methods, including flow cytometry and the like.

Using these methods, cells can be tested for pre-existing multi-drug resistance, or for the development of multi-drug resistance during the course of treatment with chemotherapeutic agents.

Furthermore, the same methods can be utilized on established cell lines or even on various membrane preparations or vesicles expressing reconstituted Pgp. On these or other model systems it will be possible to assay a given agent for its predicted usefulness in overcoming MDR, or for the development and screening of potential agents that are contemplated as being useful for modulation of MDR. The idea of using chemical compounds as modulators of MDR relies on the ability of these modulators to enter the cells to be treated and to occupy the machinery of the cell that is required for drug extrusion, thereby enabling the accumulation of the anticancer agents needed for therapeutic purposes.

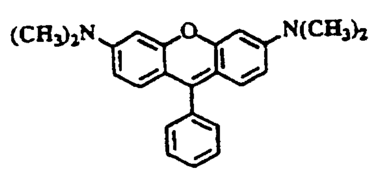
The proposed use of various compounds as Pgp modulators for the reversal of clinical muti-drug resistance is well known in the art (Sicis, 1993, J. Clin. Oncol. 11, 1629-1635). However, prognostic criteria enabling selection of agents to render MDR cells sensitive to cytotoxic drugs have not been established. The assays according to the present invention fulfill an unmet medical need in that they provide a functional assay for the prognosis of MDR reversal.

The methods of the present invention will be particularly useful to assay cells or tumor samples from a patient for the existence of multidrug resistance or its onset or development during chemotherapy. These methods will further

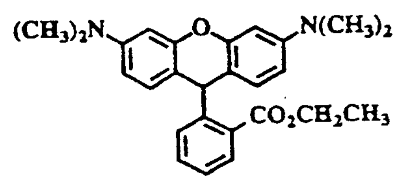
provide means for evaluating the sensitivity of tumor or cell samples to known MDR modulators or for the development of novel MDR modulators.

The principles of the present invention may be exemplified utilizing a family of related fluorescent compounds consisting of known rhodamine dyes. The structural formulate of various rhodamine dye analogs are presented in the following scheme (Scheme 1):

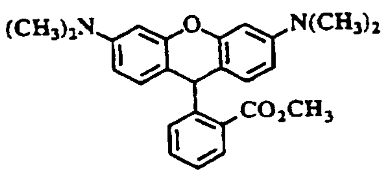
Scheme 1



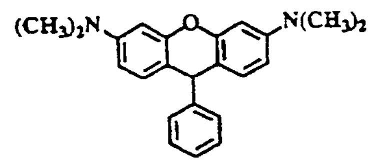
TMR



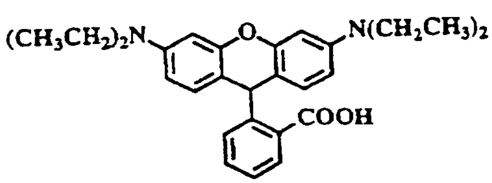
Rhodamine I



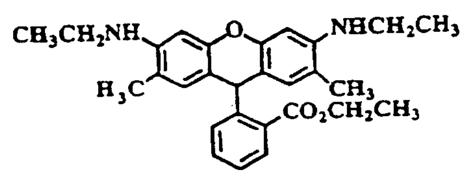
Rhodamine II



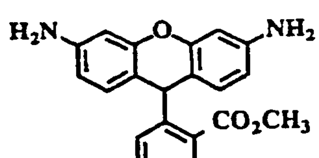
Rhodamine III



Rhodamine B



Rhodamine 6G



Rhodamine 123

This battery of known rhodamine dyes were tested for: (i) Pgp-mediated exclusion from MDR Chinese hamster ovary cells; (ii) their ability to stimulate ATPase activity of Pgp reconstituted in proteoliposomes; and (iii) their transmembrane movement rate in artificial liposomes. All the rhodamine dyes tested were substrates of both reconstituted Pgp and Pgp in viable MDR cells. Transmembrane movement rate proved the major factor correlating with the efficiency of Pgp in excluding rhodamine dyes from MDR cells. Unexpectedly, the often used MDR-marker, rhodamine 123, proved a poor cellular MDR substrate when compared to other rhodamine dyes, particularly tetramethylrosamine.

All seven rhodamine dyes studied proved Pgp-substrates as judged both by the success of Pgp to exclude them from MDR cells, and by their ability avidly to interact with Pgp and stimulate its ATPase activity in reconstituted proteoliposomes. Interestingly, rhodamine 123, the dye widely used as a functional marker of Pgp activity in MDR cells proved a poor substrate when compared to most other rhodamine dyes. Both its Pgp-mediated exclusion from MDR cells and its affinity toward reconstituted Pgp were low when compared to other rhodamine dyes, particularly tetramethylrosamine.

In an effort to define the dye characteristics allowing for Pgp to efficiently extrude rhodamine dyes and MDR- type drugs from MDR cells, the levels of dye accumulation in MDR cells were compared using the following parameters: (i) the affinity toward Pgp evident as the apparent Km of ATPase activity modulation of reconstituted Pgp; (ii) the level of maximal stimulation of Pgp ATPase activity; (iii) the level of dye binding to artificial membranes; (iv) the transmembrane movement rate; and (v) the hydrophobicity.

The best and only correlation observed was with the transmembrane movement rate. Thus, rhodamine B, which was the poorest cellular Pgp substrate, and exhibited high affinity toward reconstituted Pgp, was likewise the fastest membrane traversing dye. In contrast, tetramethylrosamine, the best cellular substrate, although, exhibiting an affinity toward reconstituted Pgp similar to rhodamine B, it was the slowest to traverse membranes among the rhodamine dyes. Clearly, Pgp plays an essential role in the MDR-phenomenon, however, the efficiency of elimination of a drug from MDR cells relies heavily on its slow rate of passive entry into the MDR cells. In this respect, only drugs with a relatively slow passive transmembrane movement rate will be efficiently extruded by Pgp from MDR cells. An anticancer drug with a fast transmembrane movement rate will overcome the MDR phenomenon and is expected to be cytotoxic even in MDR cells with Pgp overexpression.

It is now disclosed that MDR-modulators have a fast transmembrane movement rate across artificial lipid membranes, suggesting that their ability to inhibit Pgp in MDR cells is due to their relatively free entry into the cells. Since the membrane-permeable MDR-modulators are free to continuously re-enter the cells, Pgp fails to lower their concentration inside MDR cells and is thereby inhibited by them.

A better insight into the mechanism of transmembrane movement of fluorescent dyes including rhodamine dyes can be gained from the analogy to the well-characterized slow transmembrane movement of gramicidin A (O'Connell, et al., 1990 *Science*, **250,** 1256-1259). The channel-forming ionophore, gramicidin A, is an amphipathic peptide, that apart from the hydrophilic hydroxyl group it contains, it is completely hydrophobic. Gramicidin A is anchored to the membrane surface via its hydroxyl group and traverses the membrane slowly by rare flip-flop events across the membrane. Presumably, the rhodamine dyes are also amphipathic molecules possessing on the one hand, positive charges and on the other hand hydrophobic aromatic rings. Like anthracyclines (e.g., doxorubicin, De Wolf et al., 1991 *Biochim. Biophys Acta* **1096**, 67-80), the rhodamines bind very poorly to neutral vesicles, indicating that they are bound to the acidic headgroups of the phospholipids. Thus, it may be postulated that most of the rhodamine dye molecules are initially located at the surface of the membrane near the phospholipid polar headgroups. Thereafter, they slowly traverse the membrane by flip-flop events from one monolayer to the other. In this case, the transmembrane movement is best described in terms of discrete slow flip-flop events, and not by free diffusion through the membrane matrix. Irrespective of the mechanism it is clear that the selection of a fluorescent substrate having slow transmembrane movement will provide a more reliable and quantitative measure of MDR than existing assays, since it is based on a functional measurement of MDR.

An important conclusion from the slow transmembrane movement of the rhodamine dyes, applicable to all hydrophobic drugs, is that the membrane permeability properties of a drug are not determined solely by its hydrophobicity as reflected in its partition coefficient into organic solvents. Direct correlation of passive transmembrane diffusion and partition coefficient is commonly observed for a variety of small molecules. Anticancer drugs are larger molecules, positively charged, and do possess an amphipatic nature favoring their location at the membrane surface thereby interfering with rapid transmembrane movement. As such, a single hydrophilic domain can hinder transmembrane movement of a large hydrophobic molecule, e.g., the hydroxyl group in the hydrophobic peptide ionophore, gramicidin A.

The low membrane permeability of a seemingly hydrophobic dye is not limited to the MDR field and could play a major role in a wide variety of physiological and pathological situations. The intracellular concentration of a drug will be determined largely by its membrane permeability rate where its cellular concentration is a product of a competition between its passive transport and either an active efflux process or metabolic processing.

Such a case has been recently described in which a specific protein, steroidogenic acute regulatory protein, is required for the influx of cholesterol into the mitochondrion, allowing steroidogenesis to occur in the mitochondrial matrix (Lin et al. 1995 *Science*, **267**, 1828-1831). Unlike cholesterol, 25α-hydroxycholesterol readily diffuses into mitochondria, and thereby bypasses the mitochondrial cholesterol transport system. The membrane permeable 25α-hydroxycholesterol possesses two hydroxyl groups instead of one in cholesterol, and is more hydrophilic. Interestingly, the two hydroxyl groups are located at both poles of the molecule, thereby rendering the molecule less amphipathic than cholesterol.

The successful lowering of the intracellular concentration of multidrug resistance (MDR) type drugs by P-glycoprotein (Pgp) relies on its ability to overcome the passive influx rate of the drugs. The transmembrane movement of rhodamine 123, a widely used MDR-marker, proceeded with a life-time of 3 min and the transmembrane movement of various anticancer drugs occurred at a comparable rate. The present invention correlates the rate of the passive transmembrane movement of MDR substrates and the ability of Pgp to extrude them from cells. Rhodamine dyes were examined for their Pgp-mediated exclusion from MDR Chinese hamster ovary cells, their capacity to stimulate the ATPase activity of Pgp reconstituted in proteoliposomes, and their transmembrane movement rate in artificial liposomes. All rhodamine dyes tested were substrates of both reconstituted Pgp and cellular Pgp. The transmembrane movement rate proved the major factor determining the efficacy of the Pgp-mediated exclusion of rhodamine dyes from MDR cells. Thus, rhodamine B, the poorest cellular substrate, exhibited a high affinity toward reconstituted Pgp, was likewise the fastest membranes traversing dye. In contrast, tetramethylrosamine, the best cellular substrate, although exhibiting an affinity toward reconstituted Pgp similar to rhodamine B, was the slowest among the rhodamine dyes to traverse membranes.

Therefore, an anticancer drug with a fast transmembrane movement rate is expected to overcome the MDR phenomenon. Furthermore, the widely used MDR-marker, rhodamine 123, proved an inferior cellular MDR substrate

compared to other MDR rhodamine dyes, whereas tetramethylrosamine proved a superior cellular MDR substrate for functional dye exclusion studies.

As herein disclosed the selection of rhodamine or other fluorescent dyes having a comparatively slow transmembrane movement will provide a superior assay of MDR, devoid of the shortcomings of existing assays. The novel use of these slower dyes unexpectedly offers the advantage of virtually no background staining in the MDR positive cells.

Importantly, it is now disclosed that these dyes are also superior in the prediction of usefulness for anticancer drugs or for compounds useful in MDR modulation as well as for screening of cells for MDR.

The invention will better be understood with reference to the following non-limitative examples.

EXAMPLES

Materials

Rhodamine 123, rhodamine 6G, and rhodamine B base were purchased from Sigma. Tetramethylrosamine (denoted herein TMR), tetramethylrodamine ethyl ester (denoted herein rhodamine I), tetramethylrodamine methyl ester (denoted herein rhodamine II), and dihydrotetramethylrosamine (denoted herein rhodamine III) were purchased from Molecular Probes. Phosphatidylglycerol and egg phosphatidylcholine were products of Avanti Polar Lipids. The molecular formulas of the various rhodamine dyes are presented in Scheme 1 above. The FITC-labeled anti-Pgp monoclonal antibody, 4E3 and the corresponding IgG were purchased from Signet, Dedham, Ma. The FITC-labeled monoclonal antibodies, anti-CD7, anti-CD14, and anti-CD34, and their corresponding control IgG were purchased from Immuno Quality Products. Fetal calf serum (FCS) was purchased Beit-Haemek (Israel). Lymphoprep was purchased from Nycomed Pharmacia Biothech AB, Uppsala.

Experimental Procedures

Cell line cultures: Emetine-resistant cells (Emt^{R1}) derived from wild type CHO-cells using a stepwise selection protocol of increasing drug concentrations were maintained as previously described (Borgnia, M. J., Eytan, G. D., and Assaraf, Y. G. 1996, *J. Biol. Chem.* **271,** 3163-3171). T19 was a stable MDR clone isolated by a single-step selection of parental AA8 in 25 nM trimetrexate (Sharma

et al. 1991, *Cancer Res.* **51**, 2949-2959; Assaraf et al. 1994, *Anal. Biochem.* **216**, 97-109.).

Liposome preparation: Films obtained by evaporation of stock solutions containing 8 mg of phosphatidylcholine and 2 mg phosphatidylglycerol were dried under vacuum for 30 min. Multilamellar vesicles (MLV) were formed by gentle shaking in 1 ml of medium A, containing 25 mM Hepes-Tris (pH 7.5), 1 mM NaN_3, and 0.2 M NaCl, for 4 h. The vesicles were centrifuged for 2 min at 5000 rpm in an Eppendorf 5415 C centrifuge. The pellets were resuspended in medium A to a final concentration of 12.5 μM lipid-Pi. The yield of lipid-Pi was approximately 50 %.

Estimation of transmembrane movement as the equilibration rate throughout multilamellar vesicles: The rhodamine dyes tested here bind preferentially to phospholipid membranes. Upon addition of a dye to vesicles, dye binding and equilibrium with the outer monolayer is rapidly reached. Further binding and equilibration with the inner layers of multilamellar vesicles depends on the rate of transmembrane movement of the dye. Thus, apart from the initial amount of dye bound, the binding rates actually reflect the transmembrane movement rate of the dyes.

The composition of the medium in and outside the vesicles was identical and, thus, concentrative accumulation driven by electrochemical potential is not likely. Since the encapsulated volume is relatively small, the contribution of free encapsulated drug was negligible and the drug associated with the vesicles was therefore assumed to be practically all membrane-bound.

Multilamellar vesicles (1.25 mM lipid-Pi) were preincubated in 60 % sucrose in medium A. Dye was added, and after further incubation periods, 0.2 ml samples were withdrawn, layered using glass micropipettes under 0.75 ml of 40 % sucrose in medium A and 0.5 ml ether, and centrifuged for 2 min at 14000 rpm in an Eppendorf centrifuge. After gentle shaking to render the ether fraction homogenous, 0.2 ml samples were withdrawn from the ether fraction for determination of the dye amount associated with the vesicles. Total recovery of the vesicle lipids was ascertained by inclusion of N-lissamine rhodamine B-phosphatidylethanolamine (1 % of total lipids, Avanti Polar Lipids) in the vesicles and full recovery of the dye in the ether fraction. The dye amount present in the ether fraction in the absence of vesicles did not exceed 5 % of the total and was subtracted as background values.

The amount of the tetramethylrosamine, tetramethylrosamineI, tetramethylrosamineII, tetramethylrosamineV, rhodamine 123, rhodamine 6G, and rhodamine B was determined as the fluorescence (excitation 555, 556, 555, 530,

504, 530, and 558 nm; emission 575, 574, 573, 548, 525, 530, and 558 nm respectively) of ethanol:ether (8:2) solutions.

Flow cytometric analysis of rhodamine dyes uptake into cells: Cells in monolayers were first grown for 1 day in drug-free medium, then incubated at 37 ° C for 1 h in the absence or presence of the dye in the growth medium. The cells were washed with a medium containing 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, and 8.1 mM Na_2HPO_4, pH 7.5, suspended to about 10^6 cells/ml in the same medium containing 1 % fetal calf serum, and analyzed by a FACStar flow cytometer (Becton Dickinson). The dyes were excited with a 488 nm line from an argon ion laser (Coherent) and the emitted fluorescence was collected through a 630-nm long pass filter.

Miscellaneous procedures: Pgp was extracted from Emt^{R1} microsomes, and reconstituted into proteoliposomes as previously described (Borgnia et al. 1996, ibid.; Eytan et al. 1994, *J. Biol. Chem.* **269**, 26058-26065). Pgp ATPase activity was determined as previously described except that inorganic phosphate was determined as the complex of phosphomolybdate and malachite green (Muszbek et al. 1979, *Anal. Biochemistry* **77**, 286-288). Background values were obtained with samples incubated on ice and were routinely subtracted from the measurements. Equilibrium dialysis of drugs was carried out at room temperature for 5 days with 100 volumes of 10 μM rhodamine dye in medium A. Dye concentrations were determined as detailed above.

Preparation and flow cytometry of cell samples: Peripheral blood samples of healthy donors were obtained from the local blood-bank. Bone marrow aspirates of peripheral blood samples were obtained from a total of 37 leukemia patients: 17 acute myeloblastic leukemia (AML), 12 acute lymphoblastic leukemia (ALL) and 8 chronic myelocytic leukemia (CML) patients. Informed consent was provided according to the Declaration of Helsinki. The diagnosis of AML and ALL was established according to immunophenotypic, morphologic, and karyotypic analyses in each patient. The diagnosis of CML was based on typical morphology of the peripheral blood and bone marrow and confirmed with cytogenetic and molecular analyses (Bennett et al. The chronic myeloid leukemias: guidelines for distinguishing chronic granulocytic, atypical chronic myeloid, and chronic myelomonocytic leukemia. Proposals by the French-American-British Cooperative Leukemia Group. Br *J Haematol* 1994, **87**, 746-54) documenting the presence of the Philadelphia chromosome (Ph) or breakpoint cluster region (bcr-ebl).

Control samples consisting of 15 peripheral blood samples were obtained from the local blood-bank. Mononuclear (MN) cells were separated from these

samples by Ficoll-Hypaque gradient centrifugation. The MN cells were either assayed immediately or cryopreserved for future analysis. Cells used immediately were suspended in RPMI 1640 supplemented with 5 % FCS. For cryopreservation, cells suspended in 10 % dimethyl sulfoxide (DMSO) and 90 % FCS were frozen slowly in liquid nitrogen. Prior to assay, frozen samples were rapidly thawed at 37 °C and diluted 10-fold with prewarmed RPMI 1640 medium containing 40 % FCS and 2 mM EDTA. The cells were washed with 10 % FCS and 1 mM EDTA in PBS, suspended in RPMI 1640 supplemented with 5 % FCS and 5 mM $MgSO_4$ to a final cell concentration of 10^6 cells/ml. The cells were preincubated for 1 hour at 37 °C, different concentrations (2.5 nM- 25,000 nM) of TMR dye were added, and the cells were then incubated again for an hour. Subsequently, the cells were washed, suspended in cold PBS supplemented with 5 % FCS, and kept at 4 °C until dye uptake into the cells was monitored. Flow cytometry was performed using a Beckton-Dickinson FACS Calibur instrument with standard fluorescence filters. For each TMR stained sample, 30,000 events were recorded. The fluorescence was compared to the autofluorescence of the cells and expressed as relative fluorescence. TMR-resistance levels were calculated as 100 x $(\mathrm{Log}(F_{sample}) - \mathrm{Log}(A))/((F_{healthy}) - (A))$, where F_{sample} equals the fluorescence of the sample, $F_{healthy}$ equals the average fluorescence of cells from healthy donors, and A equals the autofluorescence of cells not-stained by TMR.

Immunostaining: Cells stained with TMR were washed by two centrifugations at 400 x g for 5 minutes and suspensions in PBS supplemented with 2 % FCS. 50 μl of 2 x 10^7 cells/ml were incubated for 30 min at room temperature in the dark with 20 μl of either the FITC-labeled monoclonal antibody or its negative control. Following the incubation, the cells were washed twice with PBS supplemented with 2 % FCS. The cells were resuspended in 1 ml PBS supplemented with 2 % FCS, kept on ice, and monitored in the flow-cytometer as soon as possible.

EXPERIMENTAL EXAMPLES

Cellular accumulation of rhodamine dyes. The ability of cellular Pgp to exclude the various rhodamine dyes was examined as the accumulation of these rhodamine analogs in MDR cells. For this purpose, we performed a flow cytometric analysis of dye accumulation in the wild type AA8 cells that express Pgp poorly and in its MDR sublines, T19 (Drori, Eytan an Assaraf, 1995, Eur. J.

Biochem. **228**, 1020-1029) and Emt^{R1} cells (Borgnia, Eytan, and Assaraf, 1996, *J. Biol. Chem.* **271**, 3163-3171), which respectively display a moderate (16-84-fold) and high (45-100-fold) level of multidrug resistance. All rhodamine dyes were at least partially excluded from these MDR cells. Surprisingly, most of the rhodamine dyes tested proved superior MDR-substrates when compared to rhodamine 123, a marker widely used in cellular dye exclusion assays aimed at monitoring MDR. Thus, at a concentration of 0.2 μM, tetramethylrosamine was completely excluded from both T19 and Emt^{R1} cells (Fig. 1).

In order to obtain a wider range of dye exclusion efficiencies and a thorough comparison among the various rhodamine dyes, cells were stained with a higher concentration (10 μM) of the rhodamine dyes posing a stringent challenge to the cellular Pgp as an efflux transporter. Under these conditions, rhodamine 123 accumulation in the MDR cells, T19 and Emt^{R1}, was reduced only by 55 and 65 %, respectively, relative to parental AA8 cells (Figs. 1 and 2). Five rhodamine dyes proved better substrates of cellular Pgp, as compared to rhodamine 123. Tetramethylrosamine proved a superior Pgp substrate in viable MDR cells; Tetramethylrosamine-labeled T19 and Emt^{R1} cells displayed a fluorescence that was essentially indistinguishable from their autofluorescence (i.e., in absence of the dye). Moreover, Emt^{R1} MDR cells effectively reduced the cellular tetramethylrosamine accumulation even at a 0.1 mM dye concentration (data not shown). Furthermore, the exclusion of tetramethylrosamine from MDR cells could be reversed by the known MDR-modulators, verapamil and cyclosporin A (Fig. 1), indicating that the dye exclusion was indeed mediated by cellular Pgp.

In order to examine the applicability of the TMR exclusion assay to human MDR cells, we have used the well established MDR human ovarian carcinoma 2780^{ADR} cell line. Fig. 3 shows that 2780 sensitive parental cells were efficiently stained at TMR concentrations as low as 10 pM. In contrast, the Pgp-overexpressing MDR 2780^{ADR} subline required four orders of magnitude higher from the MDR cells was reversed by established MDR modulators had no significant effect on TMR accumulation in the parental cell line. These results demonstrate that TMR is an extremely effective substrate for functional evaluation of the MDR-type which can serve as an assay for the MDR phenomena and its reversal by modulators.

In order to facilitate the quantitative comparison of the various rhodamine dyes, the levels of dye accumulation for these rhodamines in T19 and Emt^{R1} cells relative to parental AA8 cells are summarized in Fig. 2. The various rhodamines are presented according to the level of dye accumulation in Emt^{R1} and T19 cells. The same presentation order was kept in the following histograms.

Transmembrane movement rate of the various rhodamine dyes. We suggest that the success of Pgp in excluding drugs from MDR-cells is determined both by the rate of Pgp-mediated efflux and by the rate of the transmembrane passive influx of the drug. Therefore, we measured the transmembrane movement rate of the various rhodamine dyes in artificial lipid vesicles. Taking advantage of the quenching of rhodamine 123 fluorescence upon binding to membranes, we recently demonstrated that rhodamine 123 transmembrane movement into artificial lipid vesicles proceeded with a life-time of 3 min (Eytan, Regev, Oren, and Assaraf 1996, *J. Biol. Chem.* **271**, 12897-12902). This direct measurement of transmembrane movement rate could not be applied to the other rhodamine dyes, since their fluorescence was not quenched upon binding to membranes. Thus, the transmembrane movement rate of the various dyes was determined indirectly as the equilibration rate throughout MLV, micelles composed of a variable number of concentric membrane vesicles (Eytan, Regev, Oren, Assaraf, 1996, ibid.).

As shown by binding studies (Fig. 4), all rhodamine dyes tightly bind to MLV and the intramembranal concentration reached is very high compared to the dye concentration present in the aqueous medium. Since the volume of the aqueous medium encapsulated in the vesicles is small compared to the total volume of the medium, practically all the dye molecules present in MLV are bound to the membranes and not free in the intravesicular lumen. Upon addition of the rhodamine dyes to MLV, the dye molecules binds rapidly to the outer surface of the MLV. Subsequently, some of the dye traverses the membranes inward crossing the inner membranes in the MLV, thereby, allowing for binding of further amounts of dye molecules until total equilibration of the dye throughout the MLV is achieved. Dye diffusion into the aqueous matrix, binding, and desorption of the dyes from the membranes are too fast to be measured under our experimental procedures. Consequently, the rate of dye equilibration throughout MLV, measured as dye binding rate, reflects the transmembrane movement rate which constitutes the limiting step of the whole process. In conclusion, the dye binding rate allows a qualitative estimate of the transmembrane movement rate and a reliable comparison of the transmembrane movement rates of the various rhodamine dyes.

The transmembrane movement rate, measured as the time required for half-maximal dye binding, showed a clear inverse correlation with the efficacy of Pgp-mediated exclusion of the various rhodamine dyes from Emt^{R1} cells (compare Fig. 5 with Fig. 2). In this respect, tetramethylrosamine, the best cellular MDR substrate, exhibited the slowest equilibration rate, while the poorest cellular MDR

substrate, rhodamine B, emerged with the fastest transmembrane movement rate and rhodamine 123 exhibited the next fastest rate.

Of all the parameters measured in the present study characterizing both Pgp activity and dye transmembrane movement rate, only the latter could be correlated with the capacity of Pgp to exclude the rhodamine dyes from MDR cells. This suggests that the dye influx rate into cells plays a major role in the MDR phenomenon. Hence, a correlation could not be established between the level of dye binding to the MLV and the efficiency of Pgp-mediated dye exclusion from MDR cells (compare Fig. 4 with Fig. 2).

Stimulation of Pgp ATPase activity by the various rhodamine dyes. In order to establish a direct interaction of the various rhodamine dyes with Pgp, the latter was extracted from a microsomal fraction isolated from the highly Pgp-overexpressing Emt^{R1} cells and reconstituted into proteoliposomes (Borgnia, et al. ibid). The Pgp content in the microsomal fraction constituted 4.5 % of the total protein content. Upon reconstitution, the relative amount of Pgp was increased to 18 %, and under the assay conditions used here all the ATPase activity of the proteoliposomes was attributable to Pgp (Borgnia, et al. ibid.). As previously reported, in the absence of exogenously added drugs, Pgp displayed a prominent basal ATPase activity, presumably due to the presence of an endogenous substrate present in the reconstituted proteoliposomes (Borgnia, et al. ibid). This basal Pgp ATPase activity could be modulated by a variety of established MDR substrates including MDR-type drugs and MDR modulators. All rhodamine dyes stimulated Pgp ATPase activity (Figs. 6 and 7); the stimulation could be characterized by two parameters: the maximal level of ATPase stimulation (Fig. 7a) and the affinity of Pgp for the rhodamine dye expressed as the apparent Km, i.e., the half maximal stimulation of Pgp ATPase activity (Fig. 7b). The levels of stimulation of the Pgp ATPase activity overlapped the acceleration effect observed with a variety of established Pgp substrates (Borgnia, et al. ibid). There was no correlation between the stimulation levels of Pgp ATPase and the ability of Pgp to exclude the various rhodamine dyes from MDR cells (compare Fig. 6a with Fig. 2). This is not surprising, since a detailed study (Borgnia, et al. ibid) assessing the stimulation of ATPase activity by known MDR-drugs also failed to reveal a correlation between the level of stimulation and the resistance level observed in MDR cells. Interestingly, the affinity of Pgp for the various rhodamine dyes did not correlate with the ability of Pgp to exclude these dyes from MDR cells (Compare Fig. 7b with Fig. 2). Rhodamine 123, the dye most often used to monitor the function of Pgp in MDR cells, exhibited the lowest affinity toward reconstituted Pgp compared to the other rhodamine dyes. These dye-affinity studies were confirmed

by competition assays using valinomycin, a peptide ionophore substrate of Pgp (data not shown). Valinomycin accelerates the ATPase activity of Pgp reconstituted into proteoliposomes at least two folds (Borgnia, et al., ibid). The various rhodamine dyes that stimulate Pgp ATPase activity to a lower extent, actually inhibit the activity of Pgp in presence of valinomycin, in accord with the competition we recently observed between known MDR-drugs and valinomycin on a common pharmacophore (Borgnia, et al., ibid).

An important characteristic of MDR-type drugs is their hydrophobicity which allows for their passive diffusion into target cells. To test whether the hydrophobicity of rhodamine dyes is the important parameter determining the efficacy of their Pgp-mediated exclusion from MDR cells, the various rhodamine dyes were examined by silica gel chromatography. As shown in Fig. 8, the hydrophobicity of the rhodamine dyes failed to show a clear correlation either with the efficacy of Pgp-mediated dye exclusion from MDR cells (compare Fig. 8 with Fig. 2) or with the transmembrane movement rates of the rhodamine dyes (compare Fig. 8 with Fig. 5).

CLINICAL EXAMPLES

TMR-resistance essays of leukemia patients. As described above, the MDR-dye, TMR, has proven a sensitive assay for MDR in cell lines. In order to test its suitability as a sensitive functional assay for MDR in clinical cases, its effect was examined on mononuclear cells from peripheral blood samples of 15 healthy donors and from patients with acute lymphoblastic leukemia (ALL), acute myeloblastic leukemia (AML), and chronic myelogenous leukemia (CML) at presentation and after chemotherapy was examined. The mononuclear cells of the leukemia patients showed a varied capacity to exclude the TMR dye, from levels comparable to the healthy donors to high levels of exclusion even at high dye concentrations.

The suitability of TMR as a functional MDR assay was demonstrated by applying it to a sample from a CML patient exhibiting low levels of MDR. As described below, rhodamine 123 efflux assay indicated that this patient's cells were not significantly more MDR-positive than cells of healthy donors.

As shown in Fig. 9, cells from this CML patient and from a healthy donor were stained with a wide range of TMR concentrations. Compared to the fluorescence of rhodamine 123, TMR fluorescence is extraordinarily high and insensitive to bleaching by light, thus, allowing reproducible assays at TMR concentrations as low as 2.5 nM. While all the cells of the healthy donor were

stained with 2.5 nM TMR (Fig 9a), most of the CML-patient cells completely excluded this concentration of the dye. Only a small subpopulation of the cells of the CML patient were stained by 2.5 nM TMR. At higher TMR concentrations, the dye penetrated into all the cells, but even at these high concentrations it was still partially excluded from most of the cells compared to the healthy donor's cells. Distinct cell subpopulations differing in their capacity to exclude TMR were observed (Fig. 9a). In contradistinction, the staining pattern of the same cells with rhodamine 123 neither revealed significant differences between the cells from the healthy donor and those obtained from the CML patient, nor distinguished between cell subpopulations (Fig. 9b).

To test the reproducibility of the TMR assay, six separate samples obtained on different occasions from the same CML patient were compared with samples from fifteen healthy donors. As shown in Fig. 10, the cellular levels of TMR obtained with the various CML samples from the same patient were very reproducible, especially at the low TMR concentrations. Multiple TMR assays of the same blood sample appeared practically identical (SD of less than 2 %, data not shown). The high reproducibility of various samples obtained from the same patient allowed for confident determinations of even low levels of TMR exclusion. Detection of low levels of TMR exclusion were most evident at 2.5 nM TMR, while the capacity to exclude high concentrations of TMR was indicative of high MDR (Fig. 10).

Comparison of the TMR assay to the currently used functional assay to the employing rhodamine 123, demonstrates the advantage of TMR. In contradistinction to the results with TMR, no significant difference was observed in the accumulation levels of rhodamine 123 between the same CML patient and healthy donors (Fig. 10).

A unique feature of the TMR assay is the distinction between cell subpopulations based on their sensitivity to this MDR-dye. TMR fluorescence is bright red and thus, can easily be distinguished from the green fluorescence of FITC-labeled monoclonal antibodies. Combination of the functional TMR assay with antibody staining allows immunophenotyping of dye-resistant cell subpopulation compared to dye-sensitive subpopulation.

This is demonstrated in Fig. 11 by double labeling of cells obtained from the CML patient described above with TMR and anti CD-34. An intermediate TMR concentration of 25 nM was chosen in order to achieve differential staining of cell subpopulations. Under these conditions TMR stained most of the cells, apart from a small subpopulation of dye-resistant cells. Comparison of the anti-CD34 stained sample with the control-IgG stained sample reveals that CD34-

positive cells are present in all the cell subpopulations defined by TMR staining.

A better display approach, as presented in Fig. 12, is based on gating the dye-resistant cells and dye most sensitive cells. The gate defining the dye-resistant subpopulation was selected to exclude practically all cells from the healthy donor, thus ensuring that all the cells from the CML patient picked by this gate are indeed TMR-resistant (Fig. 9a). This type of display shows clearly that CD34-positive and CD34-negative subpopulations are present in both dye-resistant and -sensitive cell populations. Similar double labeling and presentation approaches were employed for an anti-Pgp monoclonal antibody (Fig. 13). All the resistant cells appeared positive for Pgp. Interestingly, the dye-sensitive cells comprised of two cell subpopulations, one positive for Pgp, and the other negative. Pgp was present in the dye-subpopulations of all the patients exhibiting dye-resistance.

Occasionally, a small subpopulation of the dye-resistant cells of a patient were Pgp-negative. Presumably in these cases the dye-resistance was due to alternative pump mechanisms.

TMR-resistance assays in patients with newly diagnosed leukemia. Functional assay of MDR in leukemia patients at the initial presentation could potentially serve as an important tool in predicting the most suitable treatment course. Therefore, the TMR-resistance assay was applied to cells acquired from CML, AML, and ALL patients, both at the initial presentation and after chemotherapy. In order to maximise the sensitivity of the TMR resistance assay, the cells were exposed to 2.5 nM TMR concentration.

TABLE 1:

TMR-resistance of cells obtained from various leukemia patients.

	At Presentation	Exposed to Chemotherapy.
	% TMR-resistance	
CML	40	100
CML	44	97
CML	49	103
ALL	21	45
ALL	32	72
AML	14	14
AML	25	50
AML	28a	56
AML	52	
AML	54	
AML	75	

Cell samples obtained from patients either at initial presentation or after chemotherapy were exposed to 2.5 nM TMR. The levels of TMR resistance were calculated as described under "Materials and Methods". 100 % TMR-resistance means that the fluorescence associated with the cells is equal to the autofluorescence of the cells. 0 % TMR-resistance means that the fluorescence of the cells is equivalent to TMR-stained cells from healthy donors. The average of five cell samples obtained on different occasions. Comparison of these samples to cell samples from 15 healthy donors revealed a statistically significant difference (P=0.01).

As shown in Table 1 above, dye-resistance of cells obtained from patients at presentation varied between 14 and 54 %. In order to establish the statistical significance of these results, five cell samples were obtained on five different occasions from the same AML patient that exhibited a 28 % dye-resistance. Comparison of these dye resistance results to the data obtained from healthy donors revealed a significance value of P=0.01. Thus, TMR-resistance levels of more than 28 % are significantly different from the situation in healthy donors. Interestingly, all three CML patients, whose cells were analysed at presentation, exhibited 40-50 % dye-resistance and close to full resistance after chemotherapy (Table I and Fig. 14). Similarly, over 90 %, resistance was observed in all the eight CML cases which have been exposed to chemotherapy (data not shown). In contradistinction, AML and ALL cases exhibited varying levels of resistance both at presentation and after chemotherapy (Table 1 and data not shown).

The effect of freezing on TMR staining. All the data described above were obtained with frozen cells. The TMR staining pattern of frozen cells acquired from both healthy donors and leukemia patients exhibiting MDR was compared to the staining pattern of the corresponding fresh cell samples. As shown in Fig. 15, freezing and thawing had no effect on the cells of the healthy donor and reduced the TMR exclusion capacity of the MDR cells only when measured at high TMR concentrations.

While the invention has been described with respect to a limited number of embodiments, it will be appreciated that many variations, modifications and other applications of the invention may be made.

WHAT IS CLAIMED IS:

1. An assay for multidrug resistance of cells comprising:
(a) exposing the cells to a fluorescent dye having slow transmembrane movement;
(b) quantifying the percentage of cells stained; and
(c) determining the extent of staining of individual cells.

2. The assay of claim 1, wherein the dye has a half equilibration time through multilamellar phospholipid vesicles in excess of 10 minutes at room temperature.

3. The assay of claim 1, wherein the dye has a half equilibration time through multilamellar vesicles in excess of 15 minutes at room temperature.

4. The assay of claim 1, wherein the dye is a rhodamine dye.

5. The assay of claim 4, wherein the dye is selected from the group consisting of tetramethylrosamine, tetramethylrodamine ethyl ester, tetramethylrhodamine methyl ester, and dihydrotetramethylrosamine.

6. The assay of claim 5, wherein the dye is tetramethylrosamine.

7. The assay of claim 1, wherein the determination of cell staining is performed by means of flow cytometry.

8. An assay for selection of multi-drug resistance modulators comprising:
(a) exposing a sample of cells to the modulator;
(b) exposing the cells to a fluorescent dye having slow transmembrane movement;
(c) quantifying the percentage of cells stained, in comparison to another sample of the same cells in the absence of the modulator, and
(d) selecting a modulator capable of rendering the cells sensitive to the dye.

9. The assay of claim 7, wherein the dye has a half equilibration time through multilamellar phospholipid vesicles in excess of 10 minutes at room temperature.

10. The assay of claim 7, wherein the dye has a half equilibration time through multilamellar vesicles in excess of 15 minutes at room temperature.

11. The assay of claim 7, wherein the dye is a rhodamine dye.

12. The assay of claim 10, wherein the dye is selected from the group consisting of tetramethylrosamine, tetramethylrodamine ethyl ester, tetramethylrhodamine methyl ester, and dihydrotetramethylrosamine.

13. The assay of claim 11, wherein the dye is tetramethylrosamine.

14. The assay of claim 8, wherein the cell staining is determined by means of flow cytometry.

Fig. 1A

0.2 µM TMR

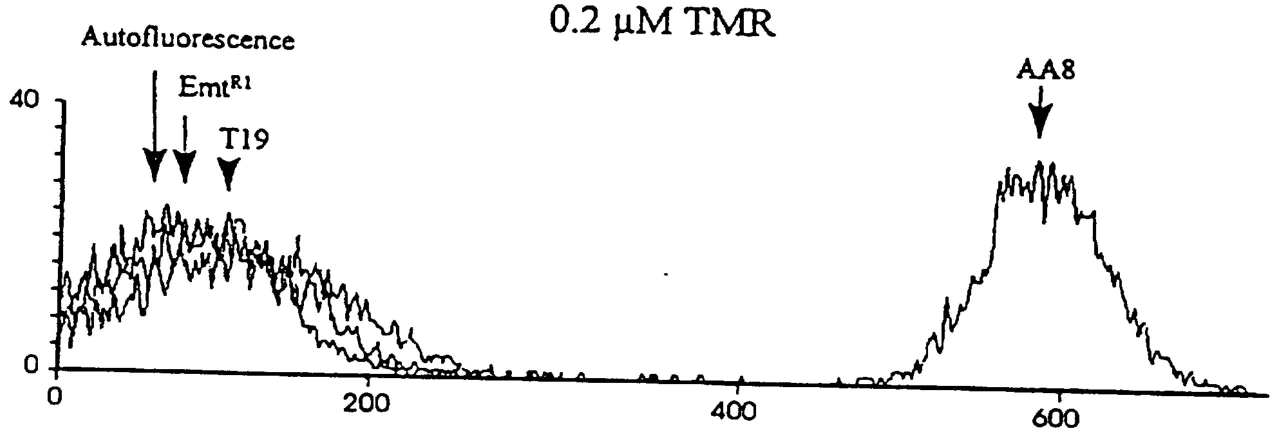


Fig. 1B

0.2 µM TMR

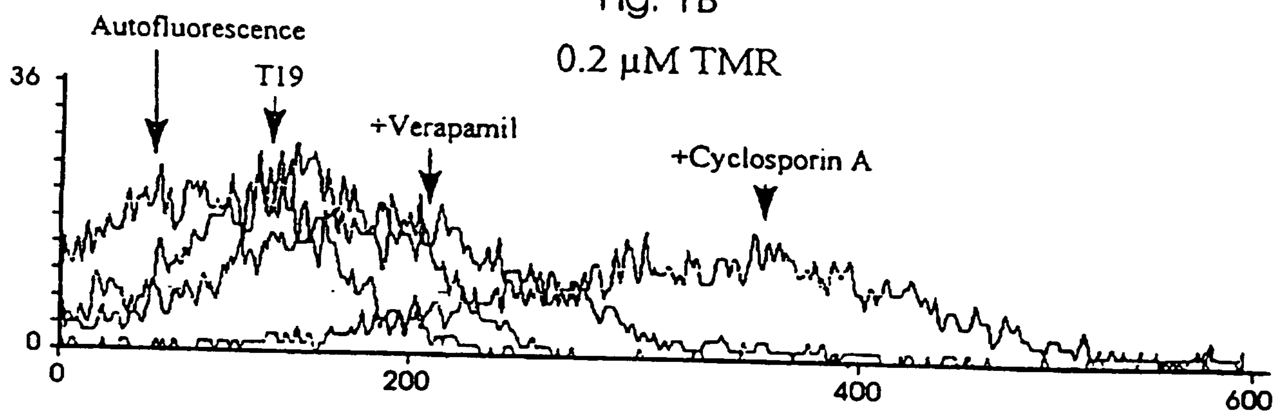


Fig. 1C

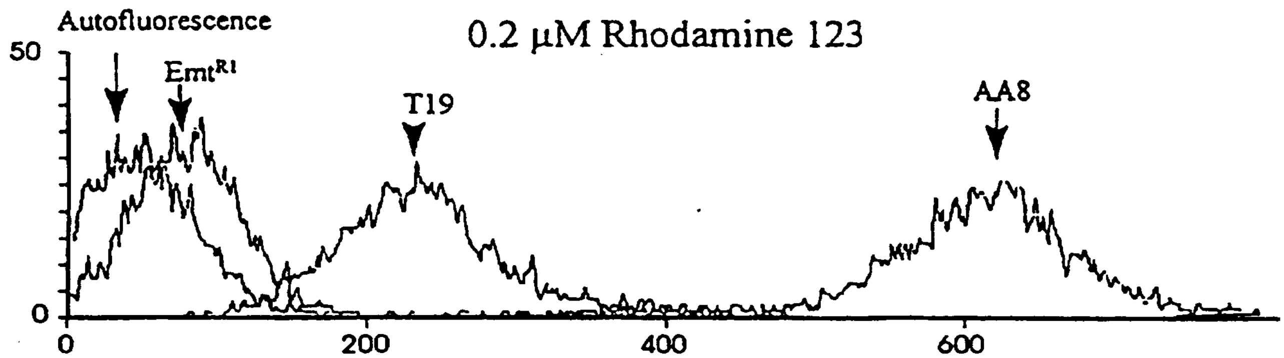

Autofluorescence
0.2 μM Rhodamine 123
50
Emt^R1
T19
AA8
0
0
200
400
600

Fig. 1D

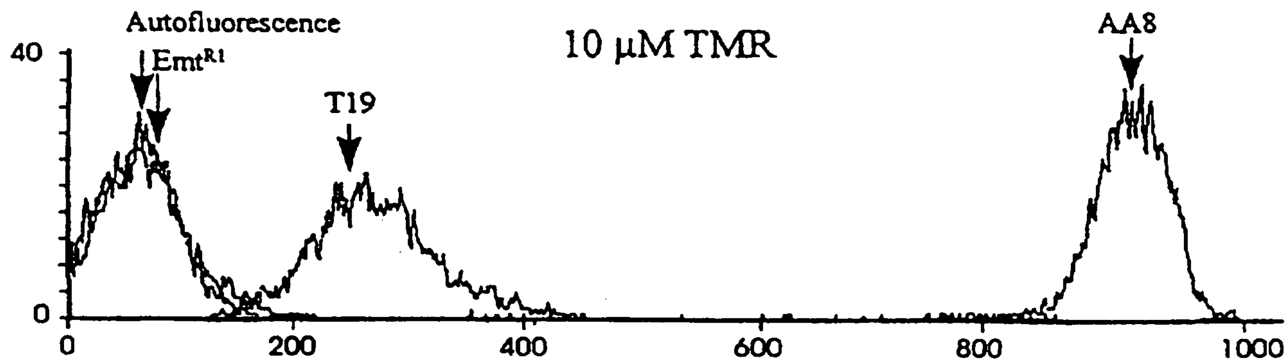

Autofluorescence
10 μM TMR
40
Emt^R1
T19
AA8
0
0
200
400
600
800
1000

FIG. 2

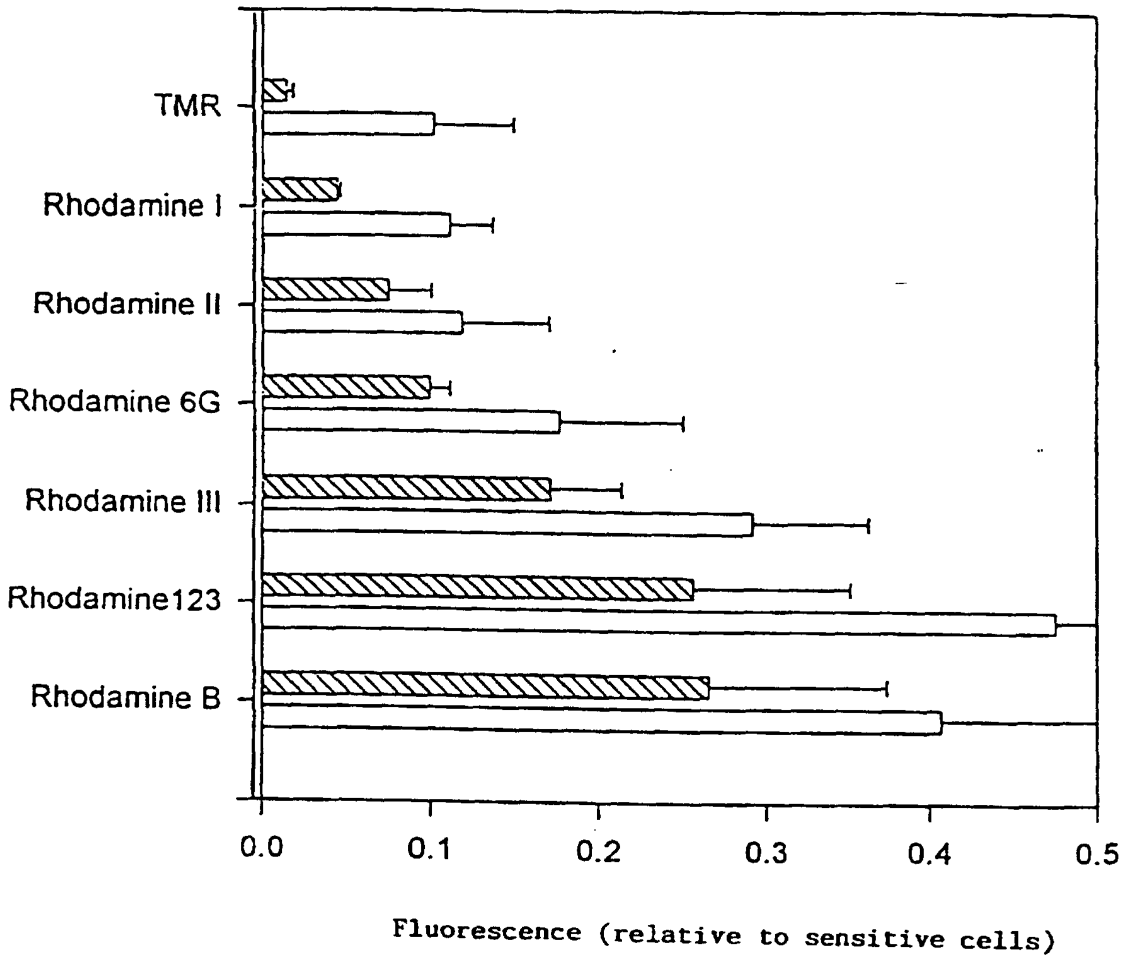

TMR
Rhodamine I
Rhodamine II
Rhodamine 6G
Rhodamine III
Rhodamine123
Rhodamine B
0.0
0.1
0.2
0.3
0.4
0.5
Fluorescence (relative to sensitive cells)

FIG. 3

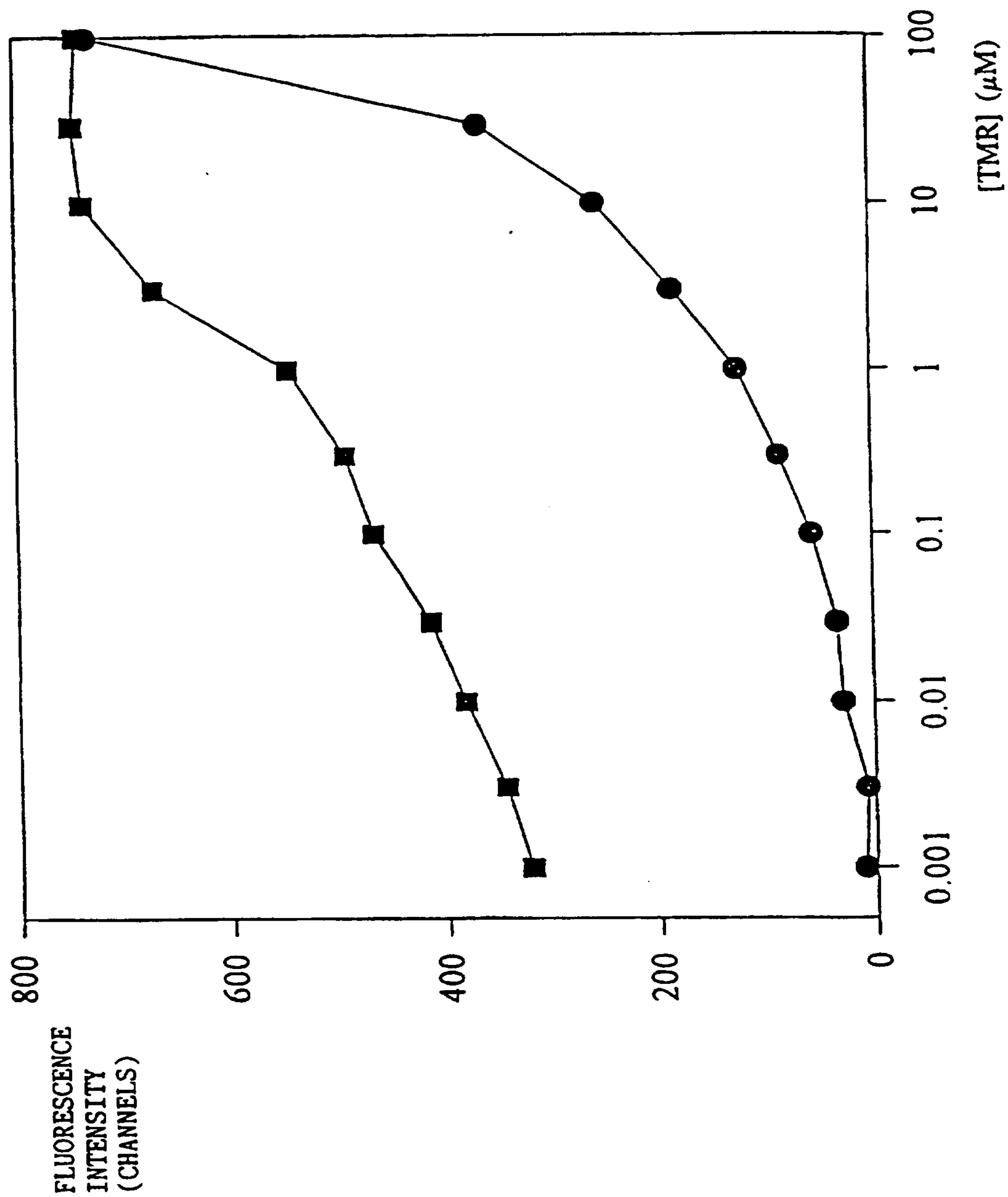

FLUORESCENCE
INTENSITY
(CHANNELS)
800
600
400
200
0
0.001
0.01
0.1
1
10
100
[TMR] (μM)

FIG. 4

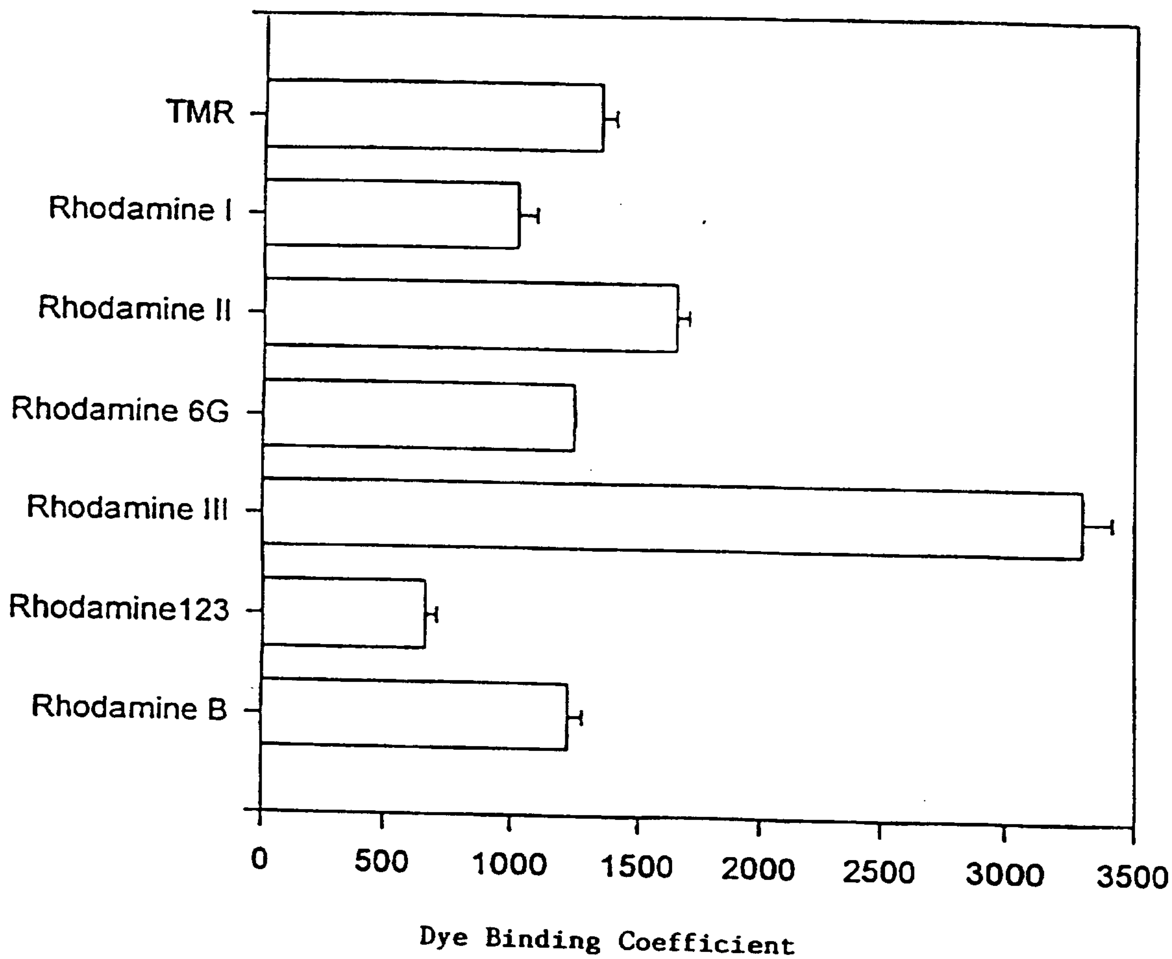

TMR
Rhodamine I
Rhodamine II
Rhodamine 6G
Rhodamine III
Rhodamine123
Rhodamine B
0
500
1000
1500
2000
2500
3000
3500
Dye Binding Coefficient

FIG. 5

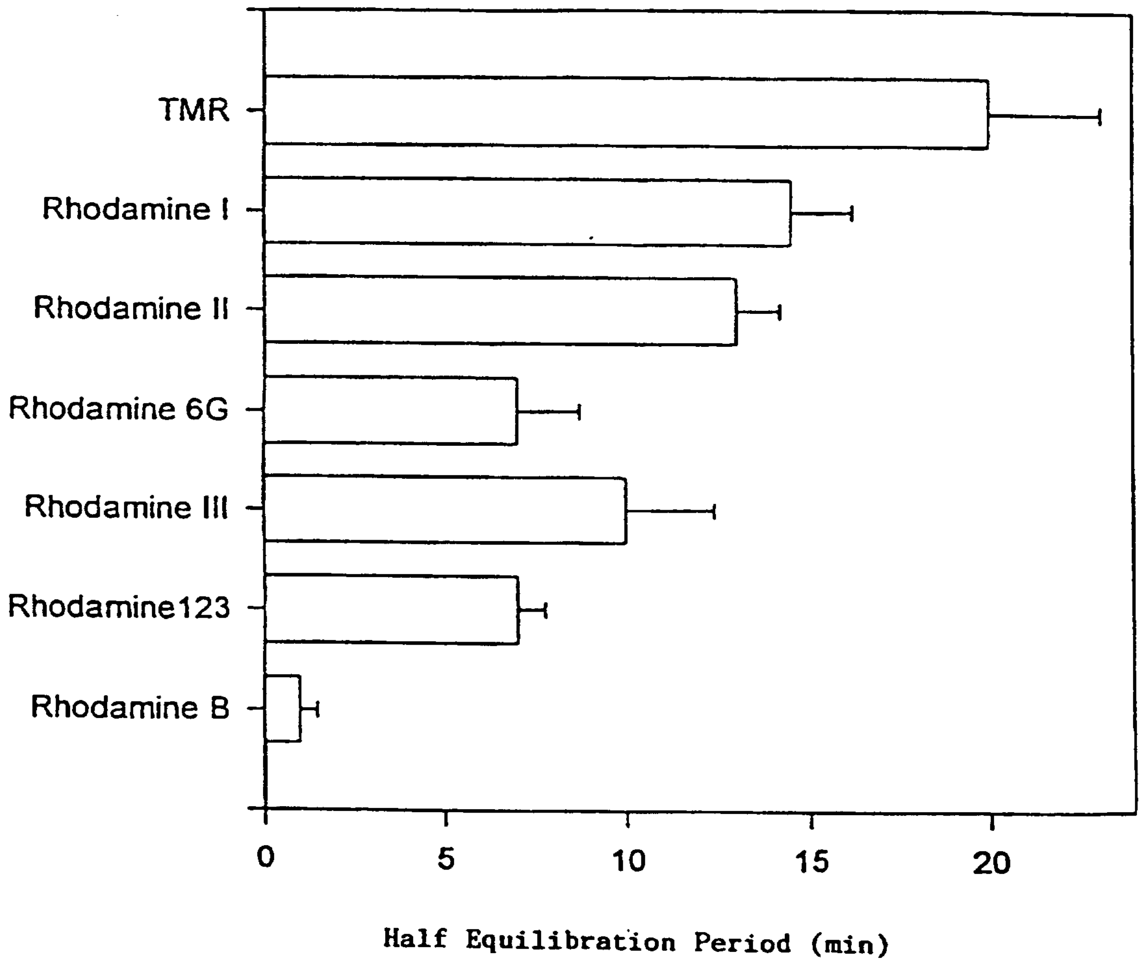

TMR
Rhodamine I
Rhodamine II
Rhodamine 6G
Rhodamine III
Rhodamine123
Rhodamine B
0
5
10
15
20
Half Equilibration Period (min)

FIG. 6

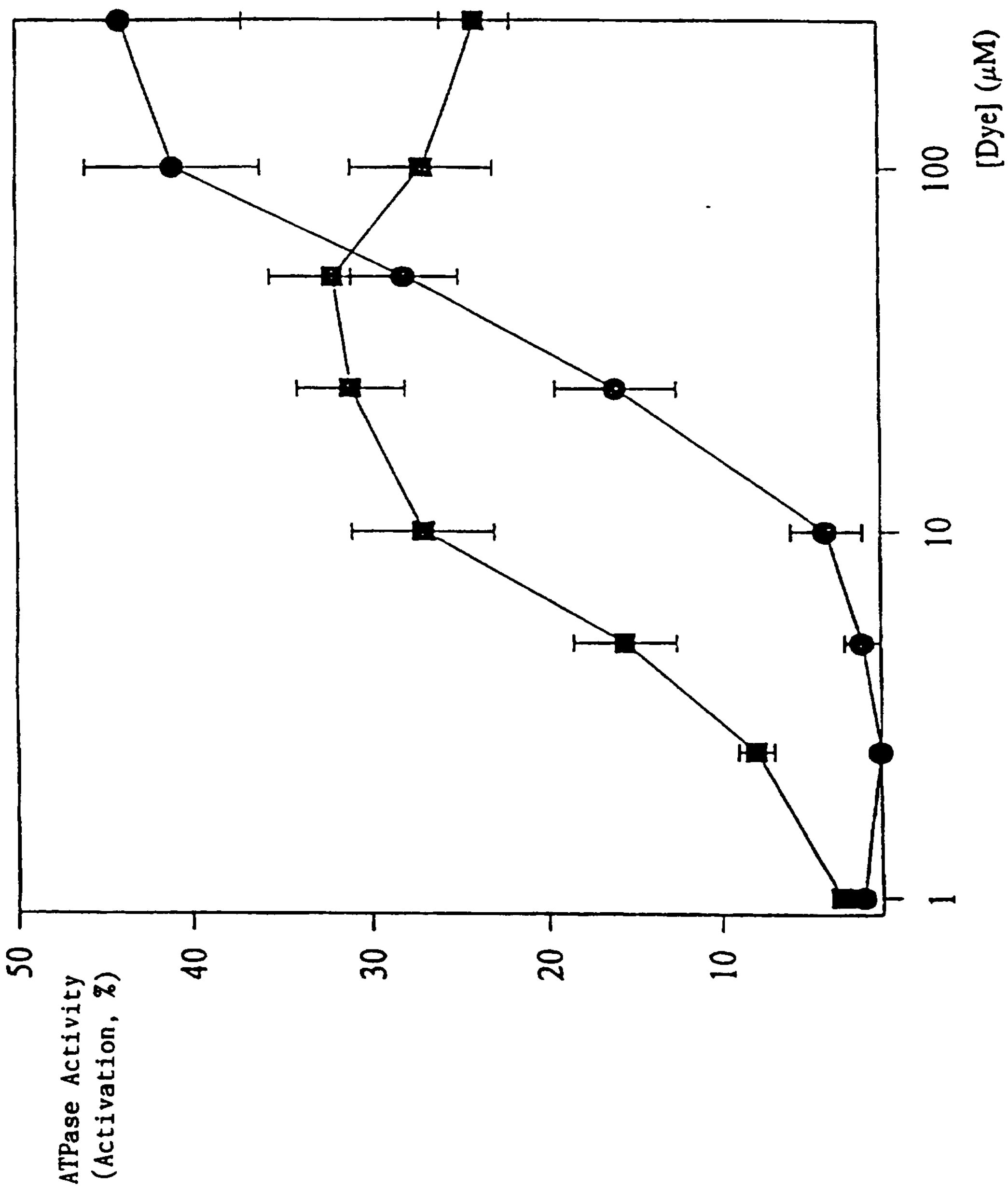


FIG. 7A

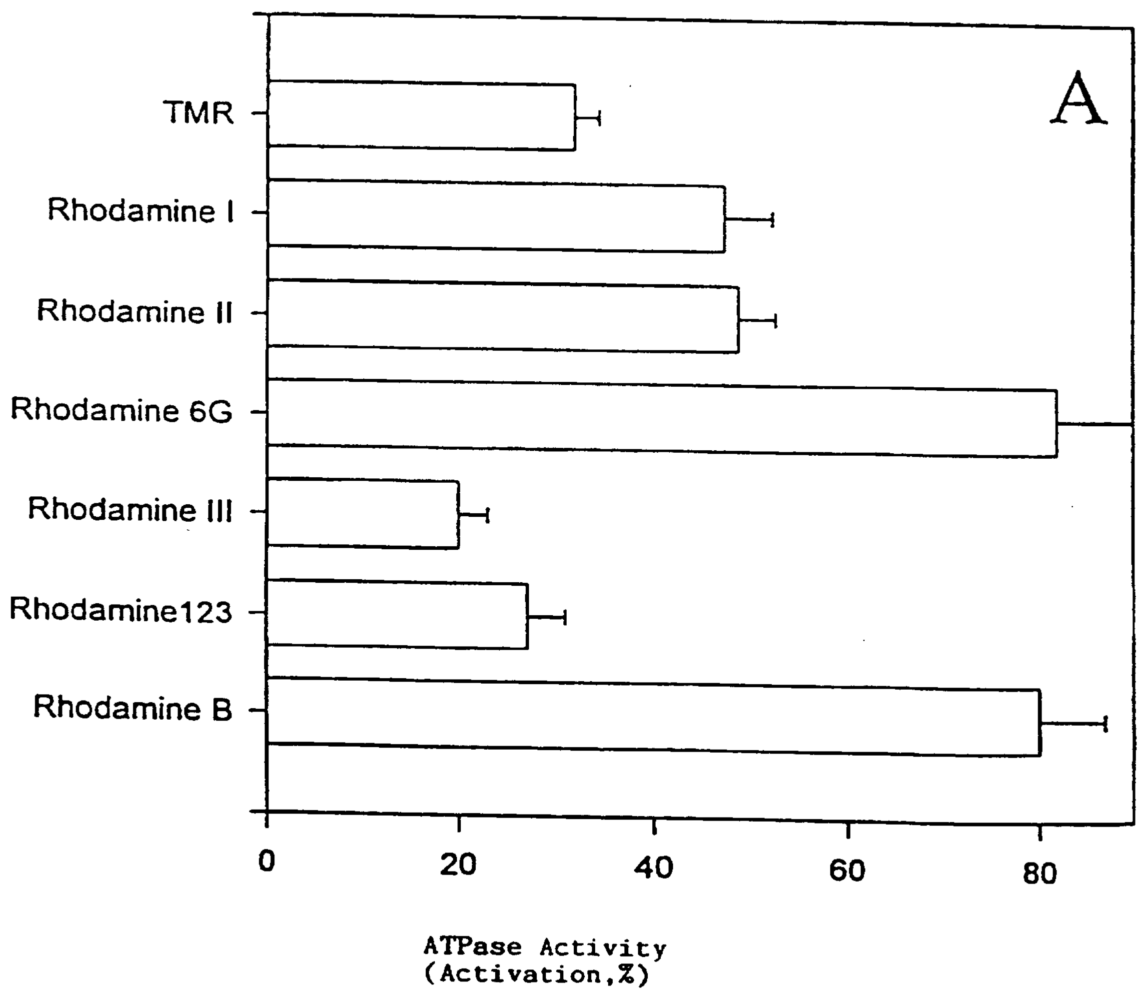

A
TMR
Rhodamine I
Rhodamine II
Rhodamine 6G
Rhodamine III
Rhodamine123
Rhodamine B
0
20
40
60
80
ATPase Activity
(Activation,%)

FIG. 7B

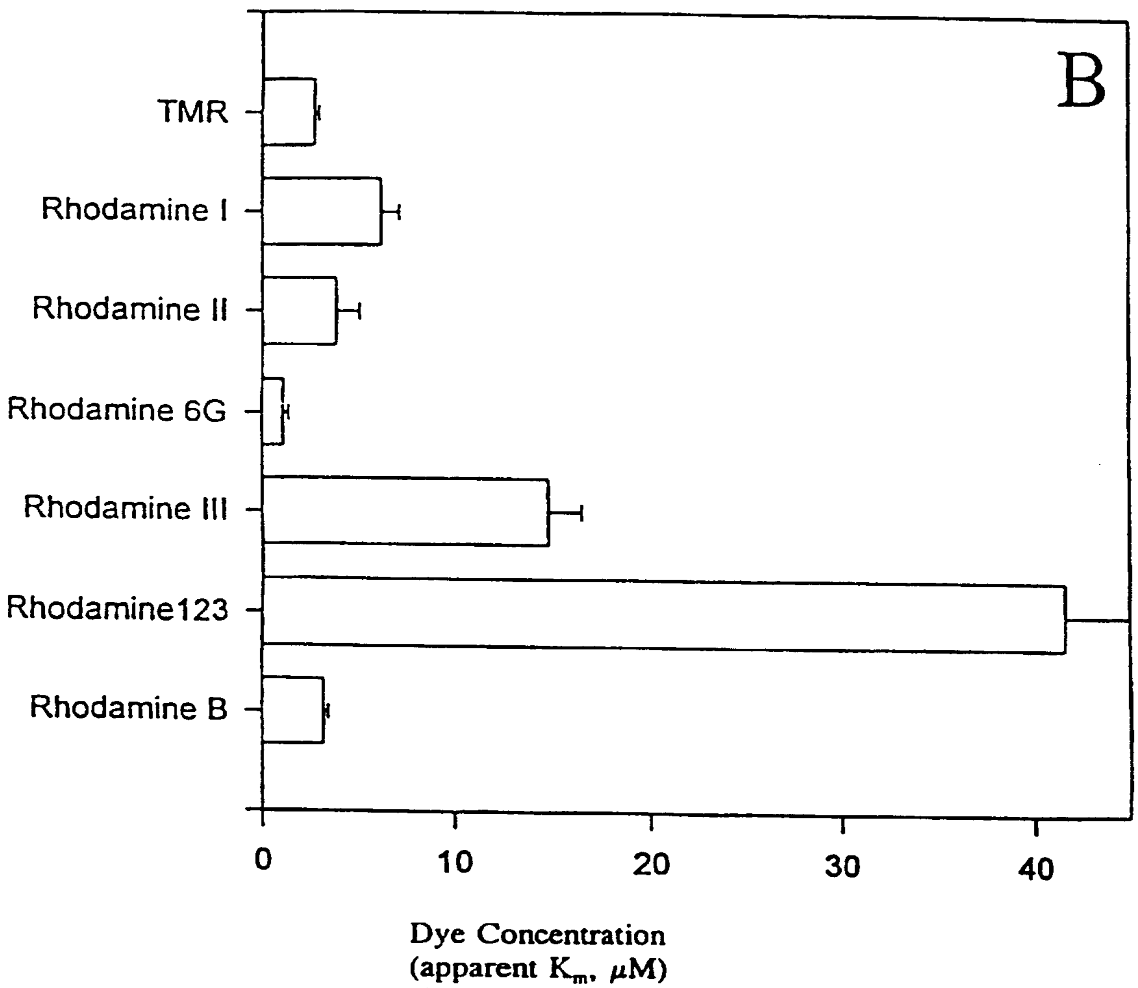

B
TMR
Rhodamine I
Rhodamine II
Rhodamine 6G
Rhodamine III
Rhodamine123
Rhodamine B
0
10
20
30
40
Dye Concentration
(apparent K_m, μM)

FIG. 8

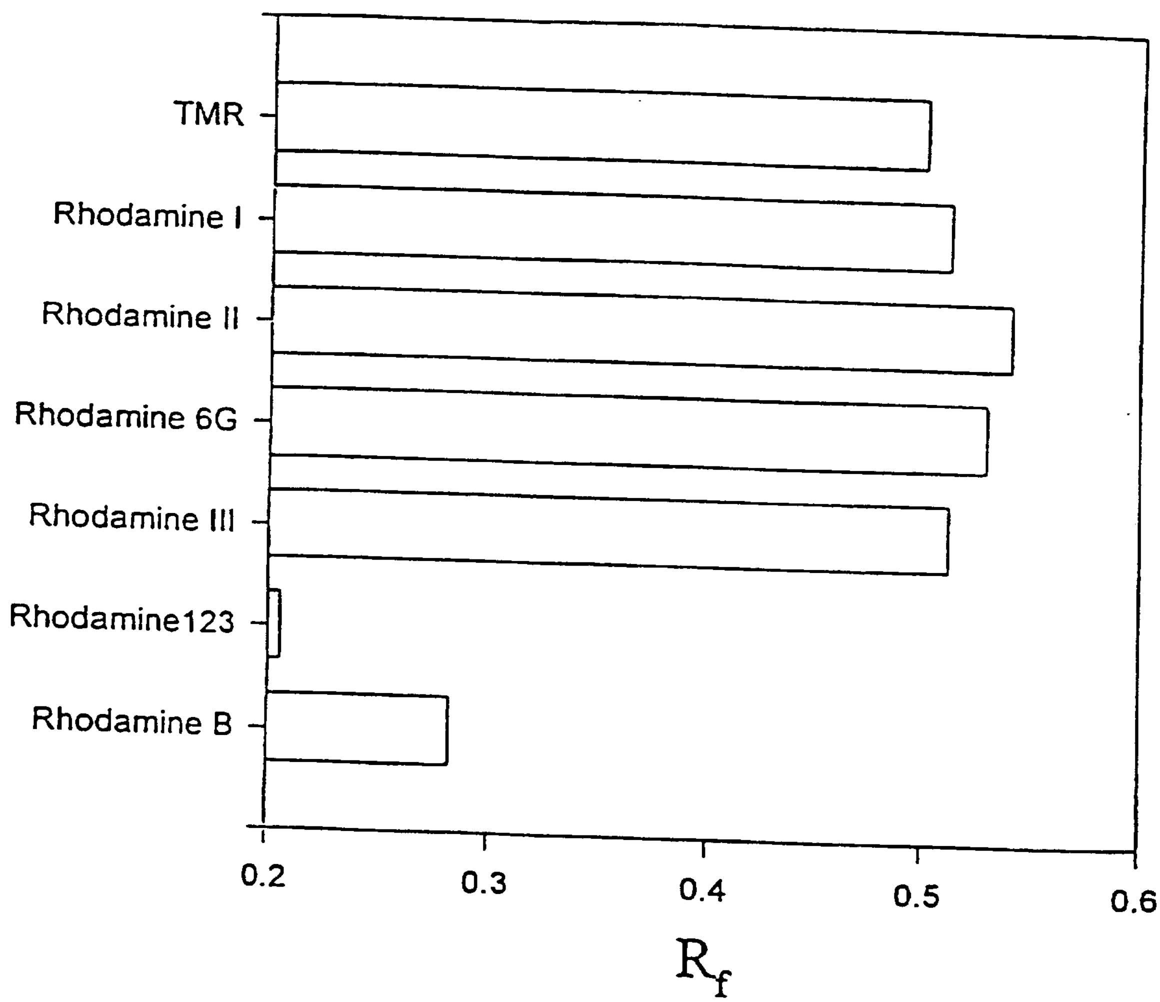
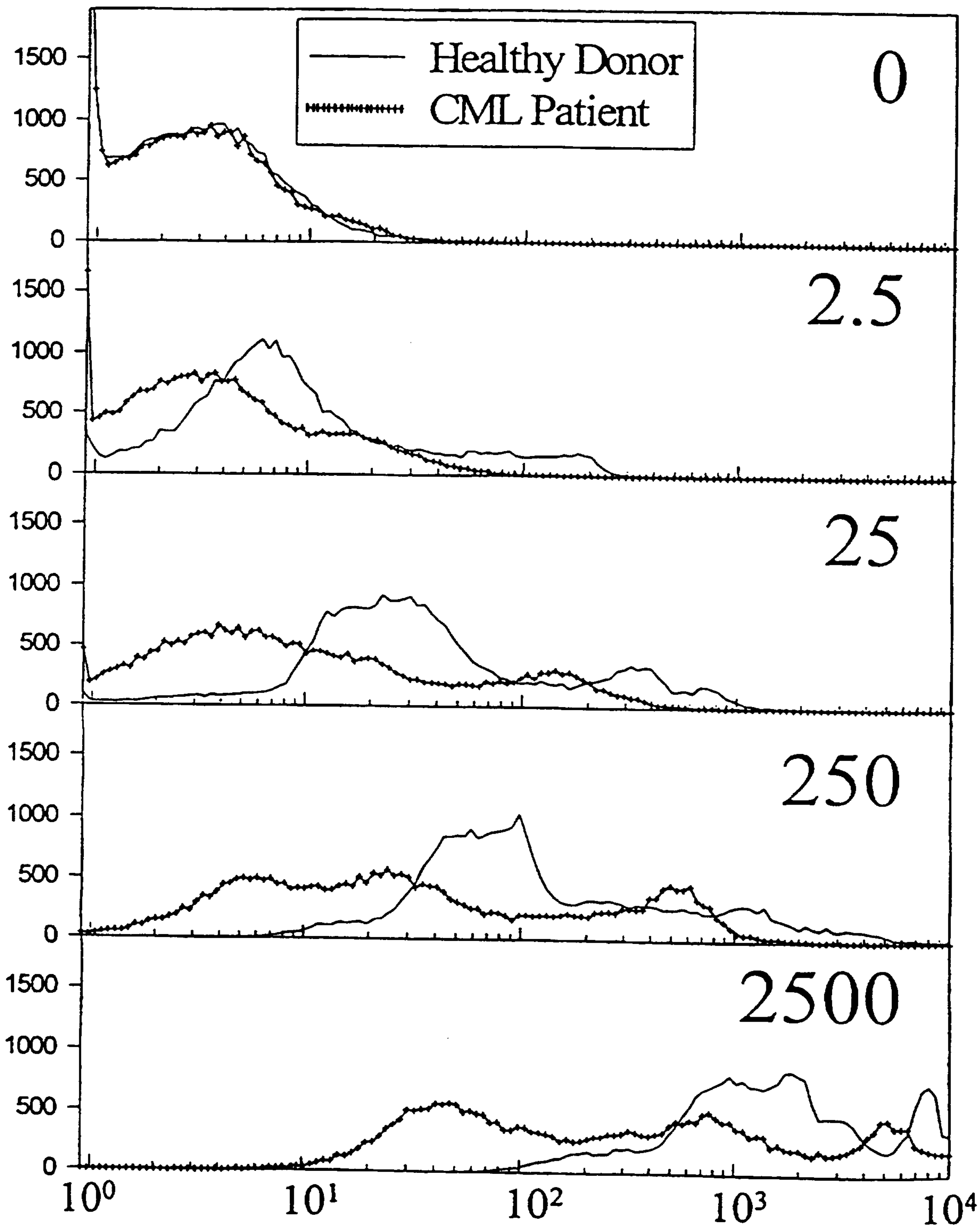

TMR
Rhodamine I
Rhodamine II
Rhodamine 6G
Rhodamine III
Rhodamine123
Rhodamine B
0.2
0.3
0.4
0.5
0.6
R_f

FIG. 9A



TMR Fluorescence

FIG. 9B

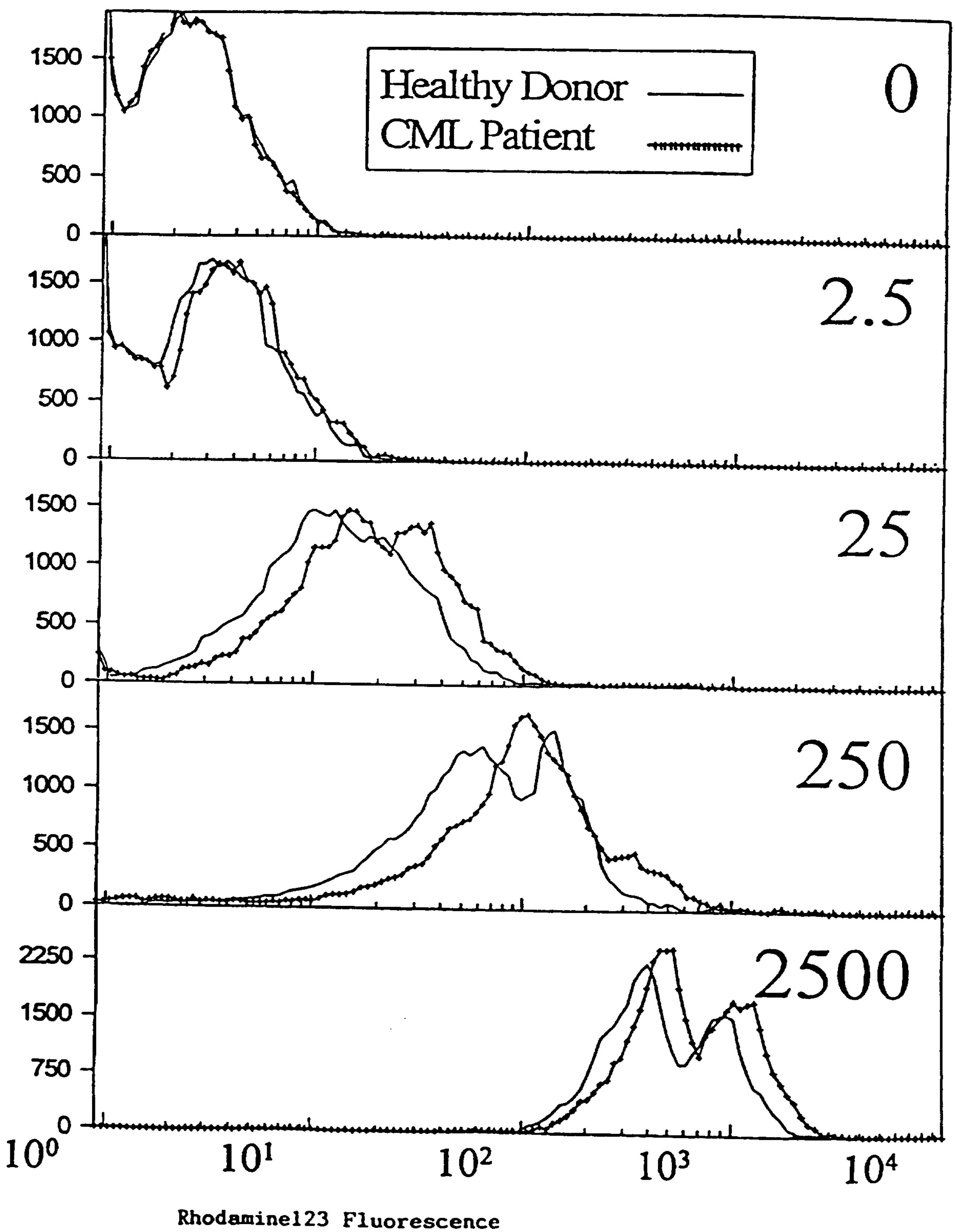

Healthy Donor
CML Patient
0
2.5
25
250
2500
1500
1000
500
0
2250
750
10^0
10^1
10^2
10^3
10^4
Rhodamine123 Fluorescence

FIG. 10 A

FIG. 10 B

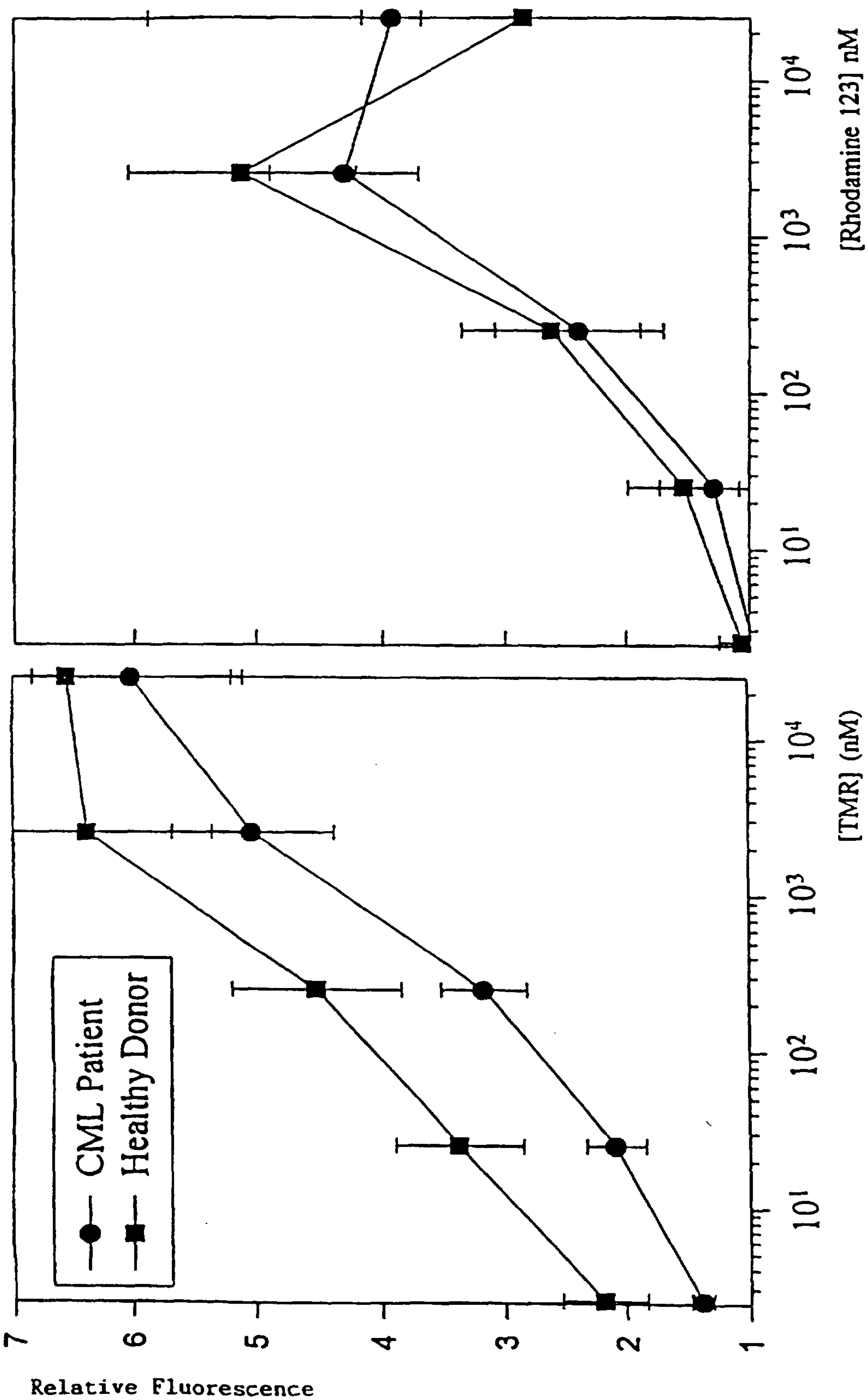


FIG. 11

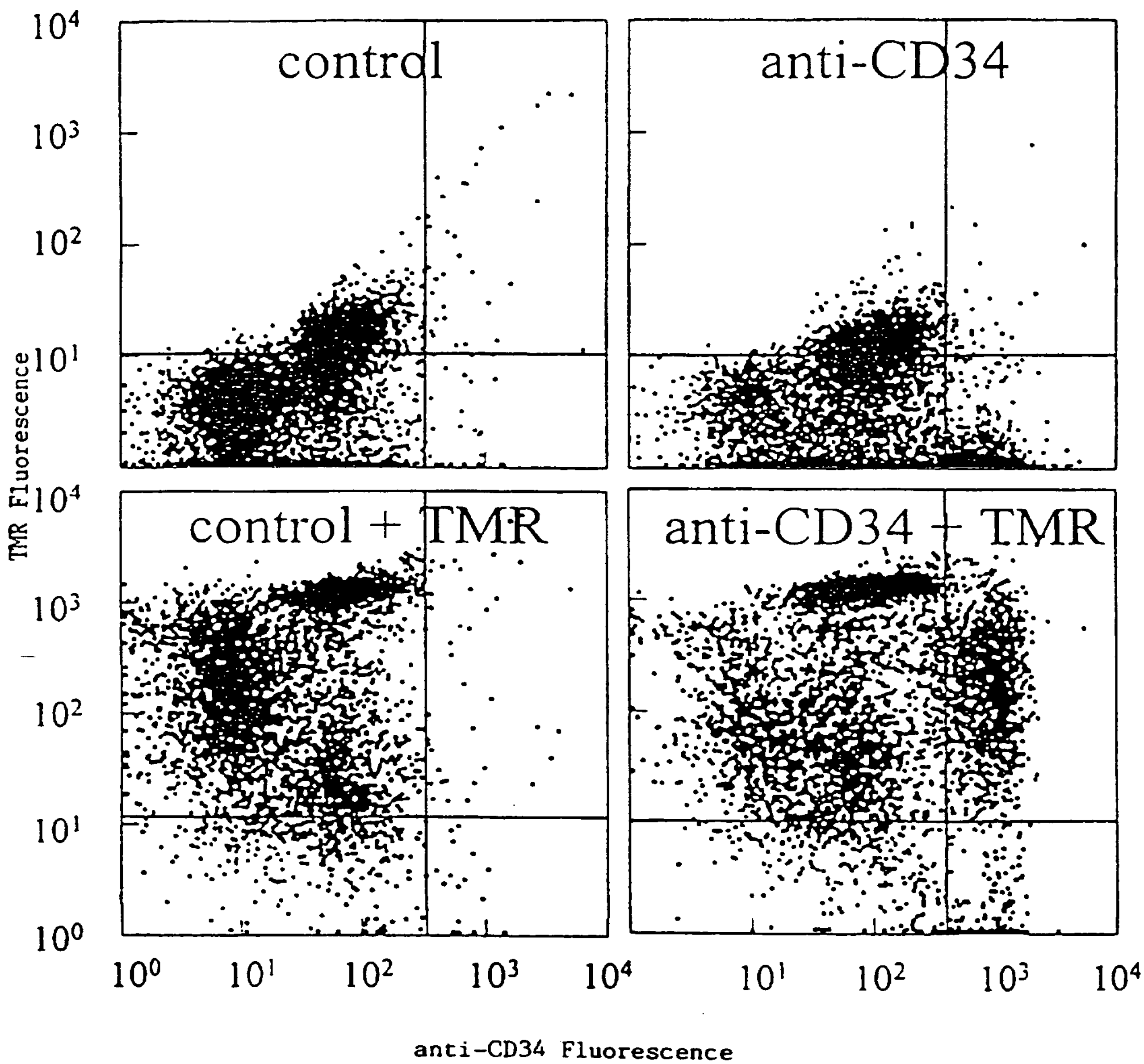

control
anti-CD34
control + TMR
anti-CD34 + TMR
TMR Fluorescence
10^4
10^3
10^2
10^1
10^0
anti-CD34 Fluorescence

FIG. 12

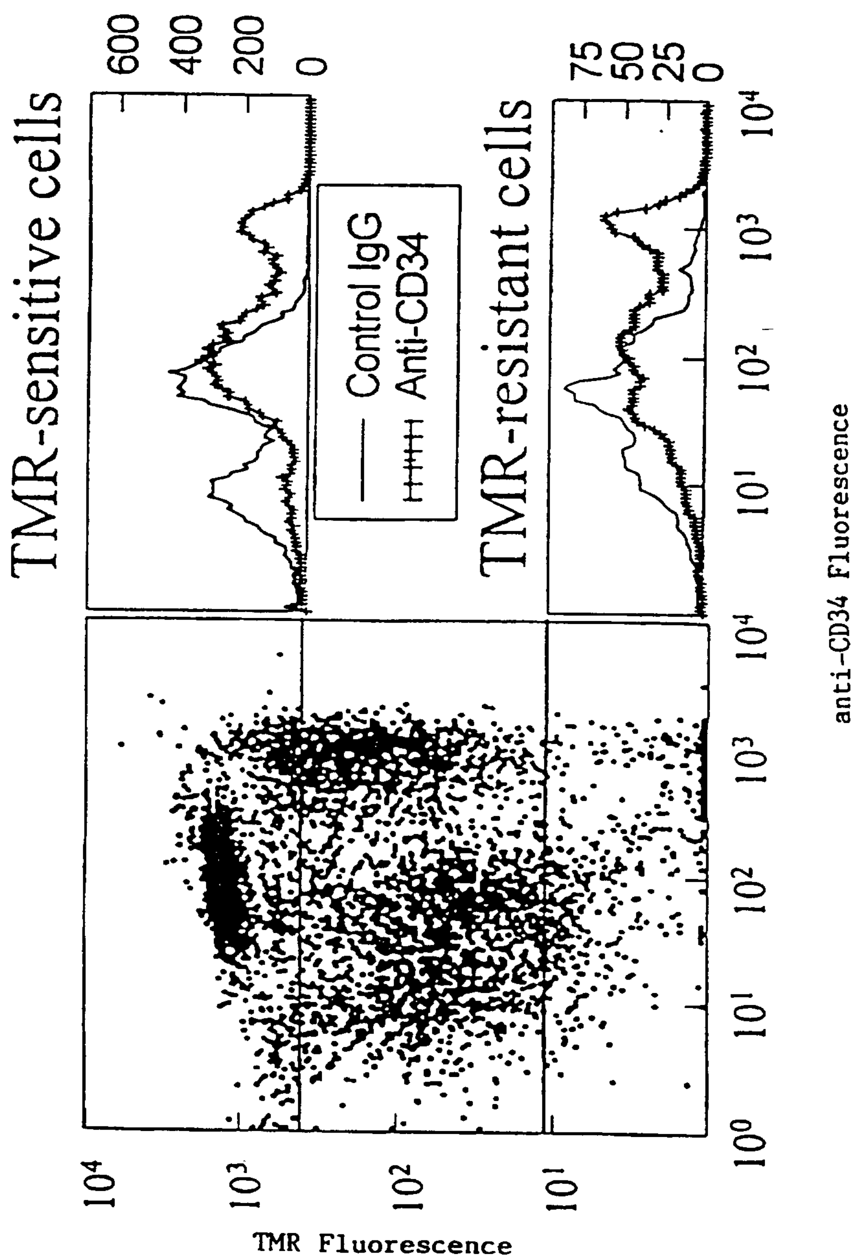

TMR-sensitive cells
600
400
200
0
Control IgG
Anti-CD34
TMR-resistant cells
75
50
25
0
10^4
10^3
10^2
10^1
10^0
TMR Fluorescence
anti-CD34 Fluorescence

FIG. 13

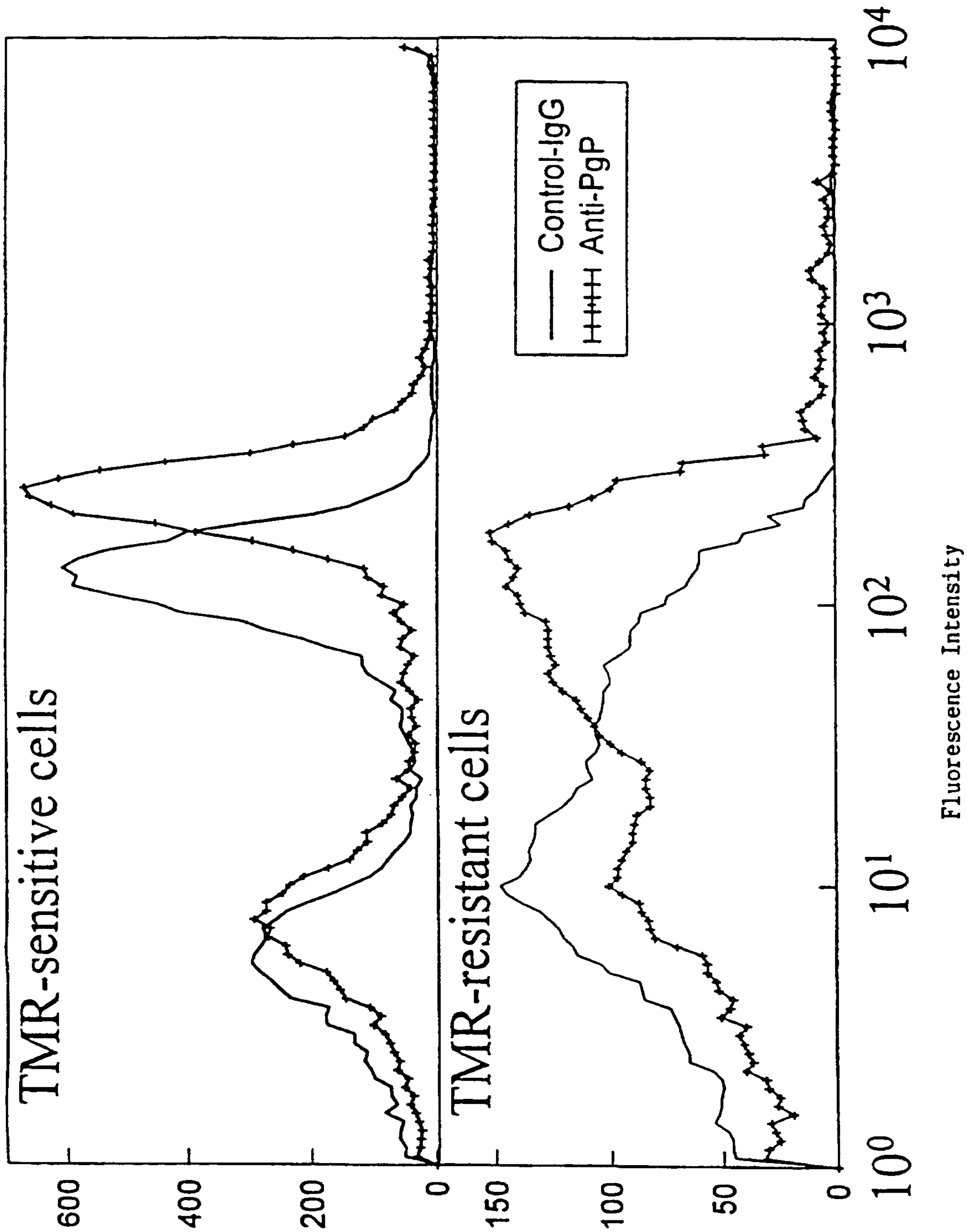

TMR-sensitive cells
TMR-resistant cells
Control-IgG
Anti-PgP
600
400
200
0
150
100
50
0
10^0
10^1
10^2
10^3
10^4
Fluorescence Intensity

FIG. 14

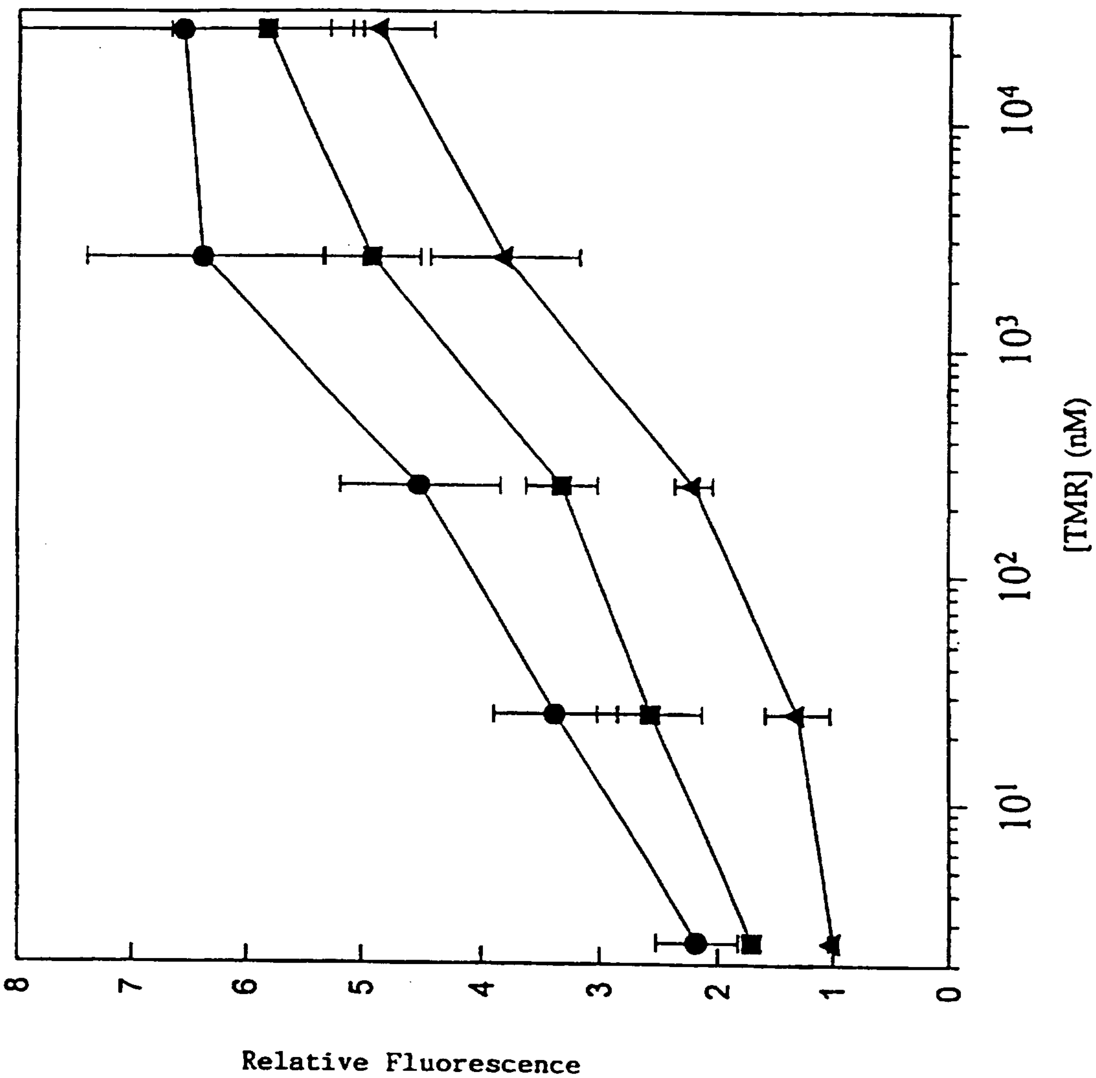
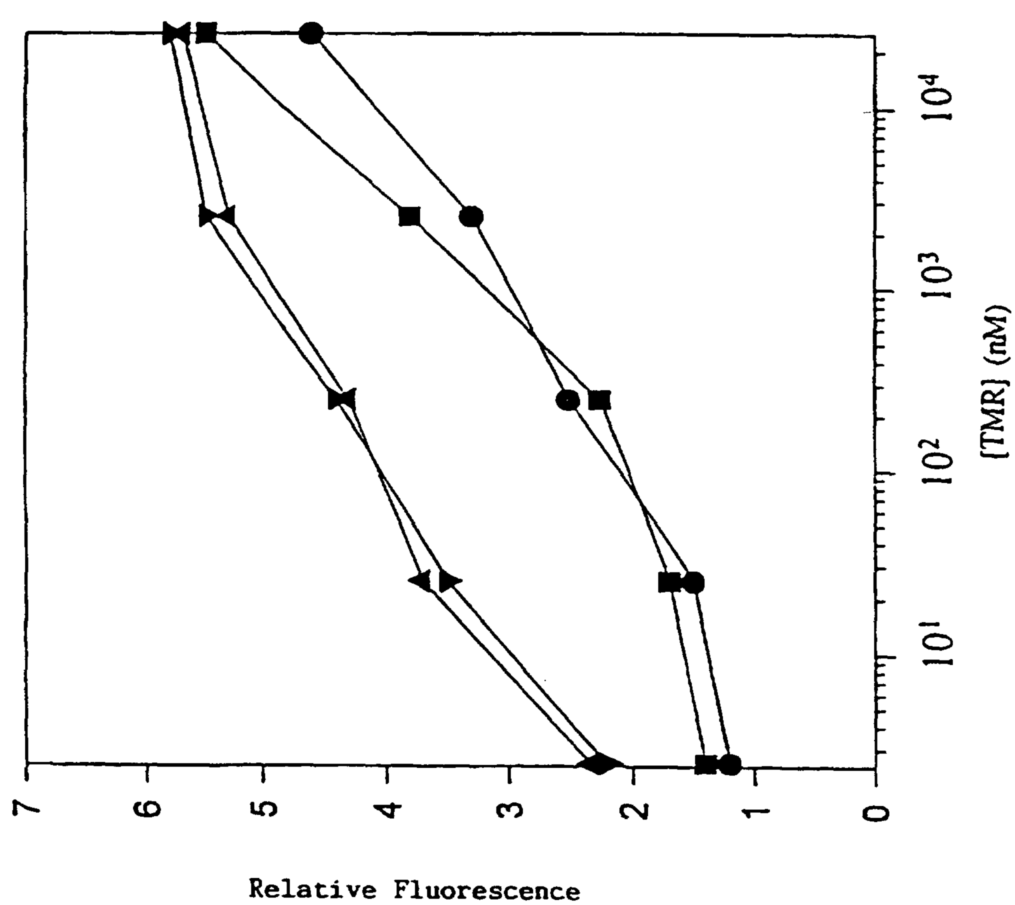


FIG. 15



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/14083

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :G01N 33/574, 33/573, 33/53
US CL : 435/7.23, 7.4, 7.9, 29, 39
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.23, 7.4, 7.9, 29, 39

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

BIOSIS, EMBASE, BIOTECH ABS, WPID, DERWENT ABS, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FREY et al. Dyes Providing Increased Sensitivity in Flow-Cytometric Dye-Efflux Assays for Multidrug Resistance. Cytometry. 1995. Vol. 20, No. 3, pages 218-227, see entire document.	1-14
X	FELLER et al. Functional Detection of MDR1/P170 and MRP/P-Mediated Multdrug Resistance in Tumour Cells by Flow Cytometry. British Journal of Cancer. 1995. Vol. 72, pages 543-549, see entire document.	1-14
X	KOIZUMI et al. Flow Cytometric Functional Analysis of Multidrug Resistance by Fluo-3:a Comparison with Rhodamine-123. European Journal of Cancer. September 1995. Vol. 31A, No. 10, pages 1682-1688, see entire document.	1-14

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 SEPTEMBER 1997	22 DEC 1997

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	IW for SUSAN A. LORING
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GRULOIS et al. Multdrug Resistance in B-Cell Chronic Lymphocytic Leukemia. Acta Haematol. 1995. Vol. 94, pages 78-83, see entire document.	1-14
X	DORDAL et al. Flow Cytometric Assessment of the Cellular Pharmacokinetics of Fluorescent Drugs. Cytometry. 1995. Vol. 20, No. 4, pages 307-314, see entire document.	1-14
X	HEGEWISCH-BECKER et al. Transduction of MDR1 into Human and Mouse Haemopoietic Progenitor Cells: Use of Rhodamine (Rh123) to Determine Transduction Frequency and In Vivo Selection. British Journal of Haematology. August 1995. Vol. 90, pages 876-883, see entire document.	1-14
X	LEE et al. Rhodamine Efflux Patterns Predict P-Glycoprotein Substrates in the National Cancer Institute Drug Screen. Molecular Pharmacology. 14 November 1994. Vol. 46, pages 627-638, see entire document.	1-14
X	LUDBSCHER et al. Decreased Potency of MDR-Modulators under Serum Conditions Determined by a Functional Assay. British Journal of Haematology. 1995. Vol. 91, pages 652-657, see entire document.	1-14
Y	BECK et al. Methods to Detect P-Glycoprotein-associated Multidrug Resistance in Patients' Tumors: Consensus Recommendations. Cancer Research. 01 July 1996, Vol. 56, pages 3010-3020, see entire document.	1-14
Y	US 5,455,161 A (ASSARAF et al.) 03 October 1995, especially abstract.	1-14